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FACULTY OF SCIENCE
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THE EXTERNAL MORPHOLOGY OF THE ANT-LION LARVA, *MYRMELEON SINGULARE*, WESTW, (MYRMELEONTIDAE, NEUROPTERA)

Part I. General Description, the Head and its Appendages.

P. S. Chitnis

Department of Zoology, Nowrojee Wadia College, Poona, India

THE antlions are insects belonging to the family Myrmeleontidae, Order Neuroptera. These are very common and occur abundantly in the Poona City area. A large amount of work has been done on the ant lions, most of which concerns the adults. Among those dealing with Indian forms, Needham (1909) has made notes on some Indian Myrmeleontidae. Graveley and Maulik (1911), have published notes on the development of some Indian Ascalaphidae and Myrmeleontidae. Esben-Peterson (listed in Zoological Records, 1931) has described the Myrmeleontidae of South India. Raff-Janet W. (1929) has described the life-history of *Myrmeleon inopinus* Walk. Trautmann (listed in Zoological Records, 1927) and Wheeler (1930) described the biology of ant-lions. Killington (1932) has described the life-history of *Hemerobius simulans* Walk. Peterson Alvah (1945) has discussed and illustrated some infants of insects in which he includes *Myrmeleon sp.* of Ohio.

No noteworthy attempt has been made to describe the morphology of any of the Indian ant-lion larvae. Hence an attempt has been made by the present author to describe the morphology of the larva of *Myrmeleon Singulare*, Westw occurring in Poona.

Material and Methods

The larvae were collected in the premises of the N. Wadia College, Poona 1, (India). When live ants were put in the larval pits, the ant-lion larvae came out of the soil and tried to grab the ants. Such larvae in the act of capturing the prey were caught with the forceps. The larvae were killed and preserved in Alcoholic Bouin's fluid. To study the external anatomy, they were boiled for about 15 to 20 minutes in 5% KOH solution, later washed in tap water. They were then dehydrated, cleared in clove oil and finally mounted in canada balsam. The dissections

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were performed under a binocular microscope. The drawings were made under a camera lucida.

General Description

The larva (Fig. 1) is gray to black in colour, is active and is thysanuriform. The body which is dorsoventrally flattened, is clearly divisible into head, thorax and abdomen. The head (Hd) bears a pair of long sickle-shaped mandibles. A membranous cervix (Cvx) joins the head with the thorax. The thorax (Th) is three-segmented, the prothorax being narrower while the meso-and meta-thorax are broader. Each thoracic segment bears ventrally a pair of legs. The abdomen (Abd) is eight segmented ; each segment, except the last, having a rounded bulge laterally. The first segment is the broadest, while the succeeding segments taper gradually, the last segment being the smallest and semicircular in shape.

The body is uniformly covered with well developed 'setae' and hairs. The lateral bulges of the abdomen bear a group of spine-like hairs. The terminal abdominal segment has on its margin long hairs, while on its dorsal surface there are thick tubercles arranged in two rows.

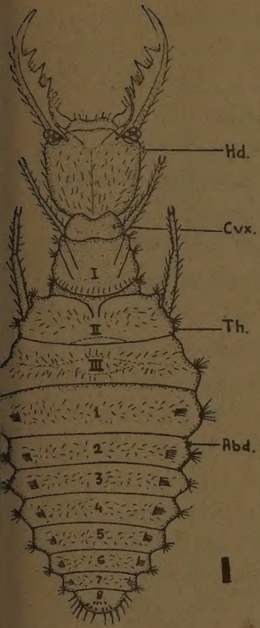
External Morphology of the Head Region

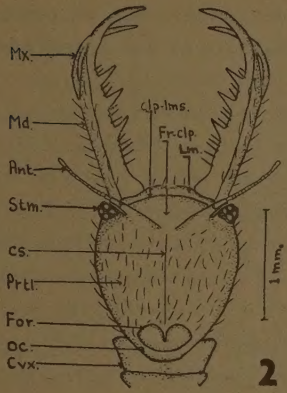
Head Capsule (Figs. 2 and 3.)

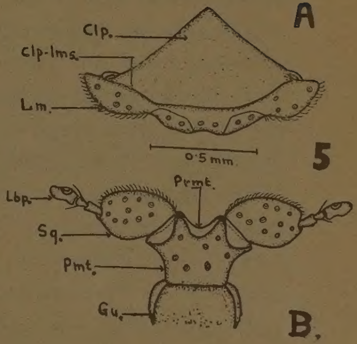
The head of the ant-lion larva is dorso-ventrally flattened and prognathous. The head capsule is oblong, being longer than broad, the length of the cranium is almost equal to the projecting mandibles and maxillae.

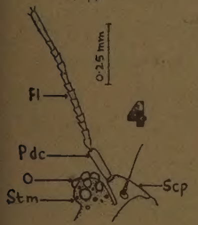
The sclerites of the head capsule are so completely fused together that it is hardly possible to see them clearly at all. Due to the prognathous nature of the head, the antennae (Ant) and the stemmata (Stm) have been shifted to the dorsal side and are situated at the anterolateral corners of the head. The fronto-clypeal area (Fr-clp) is a small triangular plate between the two antennae and has its anterior margin broader, with which articulates the labrum (Lm). Between the labrum and the fronto-clypeus is a clypeo-labral suture (clp-lms). An indistinct coronal suture (C S) runs in a median line. The parietal areas (Prtl) are large, on either side of the coronal suture. The posterior region of head, the occipital area (OC), carries dorsally the foramen magnum (For).

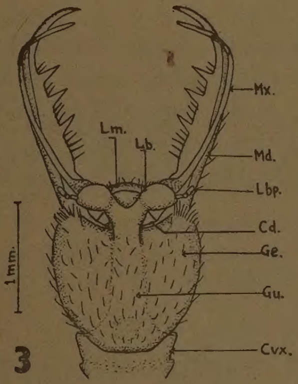
The ventral side of the head capsule is represented by the genae (Ge)











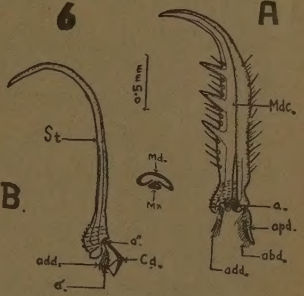


Fig. 1—Dorsal View of Larva.
Fig. 2—Dorsal View of Head.
Fig, 3—Ventral View of Head.
Fig. 4—Antenna and Stemma.
Fig. 5—A—Labrum ; B—Labium.
Fig. 6—A—Mandible ; B—Maxilla.

a, a', a'', condylar surfaces ; Abd, abdomen ; abd, abductor muscle ; add, adductor muscle ; Ant, antenna ; apd, apodeme : Cd, cardo ; Clp, clypeus ; clp-lms, clypeo labral suture ; cs, coronal suture ; Cvx, cervix ; Fl, flagellum ; Fr-clp, fronto-clypeal area ; For, foramen magnum ; Ge, gena ; Gu, gula ; Hd, head ; Lb, labium ; Lbp, labial palp ; Lm, labrum : Md, mandible ; Mdc, mandibular canal ; Mx, maxilla ; O, ocellus ; Oc, occipital area ; Pdc, pedicel ; Pmt, post-mentum ; Prmt, pre-mentum ; Prtl, parietal area ; Scp, scape ; Sq, squama ; St, stipes ; Stm, stemma ; Th, thorax.

and gular areas (Gu) which are fused together. The labium (Lb) is present on the anterior side. Brush like hairs are present on small elevations on the anterior corners of the head on its ventral side.

Antennae (Fig. 4)

The antennae are borne on the elevated areas of the dorsal side of the head capsule just inner to the stemmata. They are filiform, short, slender and 16-segmented. The scape (Scp) is cone-shaped, the basal portion being broader, bearing a long bristle. The pedicel (Pdc) is the longest segment and is attached to the scape by a membrane and is freely movable. The flagellum (Fl) is 14-segmented, each segment tapering gradually. Its segmentation is indistinct. The basal part of the first segment is narrower which articulates with the distal part of the pedicel by a ball and socket joint, giving the flagellum considerable movement in all directions, mainly along the horizontal plane. The 14th segment is the longest, club-shaped and bears 2-3 minute hairs terminally.

Stemmata (Fig. 4)

The larval ocelli are grouped together forming the stemmata. These are present on elevated areas on the dorsal side of the head capsule and are outer to the antennae. Each stemma (Stm) consists of a number of ocelli. Between the oceli (o) is present black pigment and a few hairs. The probable function of these hairs seems to be protection to the lenses from the soil particles.

Mouth Parts (Figs. 5 and 6)

The larva is predaceous and the mouth parts are adapted for grasping, injecting and sucking.

Labrum (Fig. 5 A)

The labrum (Lm) is an oblong and narrow plate anterior to the clypeus (Clp). It covers the bases of the stylets dorselly. The free side of the labrum is wavy, with two depressions and three crests. The side crests bear a number of fine hairs while such hairs are absent from the middle crest. A number of setae are also present on the dorsal surface of the labral plate.

Mandibles (Fig. 6 A)

A pair of mandibles projecting from the anterior corners of the head is the most conspicuous feature of the larva. These are very much elongated, inwardly curved, fanglike jaws. They are hinged to the anterior lateral angles of the head by the dorsal and ventral articulations. This permits movement sideways *i. e.* in a horizontal plane only.

Three regions can easily be marked in a mandible :—(1) The basal part of the mandible is lodged inside the cranium. The lateral flanges are round, while there is a notch in the midline on the posterior side. Over this notch is a squarish chitinised area which marks the condylar surface (a). A small apodeme (apd) is attached at the base towards the outer side, and the abductor (abd) muscle is inserted on this apodeme. There is no apodeme at the inner flange, but it presents a large, flat articular surface on which the adductor (add) muscle is directly attached. Minute serrated scales are present on the inner half of the base giving the surface appearance of a file. The outer half is devoid of such scales.

(2) The middle part is elongated, stout and broad. A small constriction is present between the basal and the middle part. Towards the outer margin there are two types of hairs ; the larger hairs numbering about ten and numerous smaller hairs. The inner margin shows about 11 to 13 thick, pointed, large setae. In addition, there are three very large spine-like hollow projections, and the mandibular canal extends into them. These larger spines help to hold the struggling prey.

(3) The third and distal part is a long curved, highly chitinised portion devoid of any cuticular projections. It ends in a very sharp daggerlike point which pierces through the body of the prey and the curved portion together with its counter part on the other side holds it firm like forceps. There is a row of minute serrations on the inner side of the dagger-like part.

The mandible is traversed by a tubular canal, the mandibular canal (Mdc). The canal starts proximally from the inner side of the base of the mandible at the point where the adductor muscle is attached and opens distally at the tip of the mandible. The canal extends also into the three large spines as already mentioned. On the ventral side, this canal is open and in this open groove fits the long blade of the maxilla and thus a closed channel is formed between the two appendages, through which the larva sucks the juice of its prey.

Maxilla (Fig. 6B)

Peculiar to predaceous, sucking insects, the maxillae are modified to form stylets. Each stylet has the shape of a sword, the handle of which is marked by a flat broad portion, while the blade is formed by the distal curved and slender stylet.

The maxilla is articulated with the cranium by a triangular chitinous piece which represents the cardo (Cd) of a typical maxilla. The cardo is firmly wedged between the labium and the cranial gular plate. (Fig. 3) Only the inner sides of the triangle are in contact with the labium and gular plate, while there is a narrow gap between the outer side of the cardo and the cranium. The cardo is articulated to the cranium by a small condylar projection (a') ; and towards the outer margin, the surface is marked by the line of internal ridge.

The cardo itself is formed of two sclerites which are themselves triangular in shape. The basal sclerite is smaller than the distal one. The distal sclerite has its apex pointing forwards and forming a condylar articulation (a'') to the next part, the stipes. The ventral adductor (add) muscle is inserted on the distal triangular sclerite.

The whole blade or stylet represents the stipes (St), while the maxillary palps, galea and lacinia are absent. The basal region of this stylet is broad and flat with a rounded inner border, while its outer border has a small but distinct notch-like depression in which the condyle of the distal sclerite of the cardo fits in. The whole basal region is clothed with several rows of minute serrated scales. Similar scales are also present on the basal surface of mandibles. These scales at the bases of both the mandibles and maxillae probably help to form a firm grip between the two parts.

The distal part of the stipes forms an elongated, curved blade ending in a very fine point. The margins of the blade are curved inwards a little to form a very shallow gutter towards the dorsal side. The maxilla is a little shorter in length than the mandible. The point of the maxillary blade, under high magnification shows minute serrations.

The cardo articulates with the labium (Fig. 3 Lb, Cd). The cardo is in the same plane as the labium and forms a part of the ventral side of cranium. The proximal part of the stipes is dorsal to the labium, while the remaining portion lies under the mandible close to the mandibular canal. The stipes is at right angles to the cardo making possible the horizontal movement of the blade.

Labium (Fig. 5B)

Due to the prognathous nature of the head, the labium is situated at the anterior ventral part of the cranium. It lies below the bases of the mandibles and the maxillae.

The identification of the various parts of the labium in this insect with those of a typical labium is difficult. It is marked into three different parts: (1) postmentum, (2) prementum and (3) palps.

The postmentum (Pmt) is not divisible into the submentum and the mentum. It is an entire, small, chitinised Y-shaped plate. The anterior arms of the Y are very blunt and short. The lower arm is continuous with the gular plate. A faint suture separates the postmentum from the gular plate. A highly chitinised internal ridge extends like a loop between the two extremeties of the anterior arms. A number of large setae are present on the ventral face of the postmentum.

The prementum (Prmt) is a small, crescent-shaped plate lodged between the inner sides of the anterior arms of the Y-shaped postmentum. The ligula and paraglossa are completely suppressed, like the galea and lacinia of the maxilla.

From each anterior corner of the prementum arises a very large, broad plate, the squamma (Sq). The squamma is movably articulated with the prementum, and forms the basal segment of the four-jointed labial palp. (Lbp) The sides of squammae are bulging and round. Their anterior margins only are lined with small hairs. From the surfacial area of squammae, arise a number of setae.

There is a small socket at the distal end of the squamma. From this socket arises the second joint of the labial palp. The three distal segments of the palp are small. The third segment is very small, globular and bears two large hairs. The fourth and distal segment is larger than second and third and is club-shaped. There is a peculiar pit-like mark on the antero-lateral margin of this segment, which is probably a sensorium.

The size of the labium is small compared to the mandibles and maxillae. Hence it has probaly an insignificant role in feeding, but may have a sensory function.

Summary

1. The dorso-ventral flattening, the gray to black colour and the covering of the body surface by numerous setae and hairs are all the ecological adaptations of the larva to the fossorial habit.

2. The head and the mouth parts are described in detail. The mandibles and maxillae are adapted for grasping, piercing and sucking.

The food channel is formed by the ventral groove of the mandible to which is approximated the maxilla.

3. The labium is small and may have only an insignificant role in feeding. It may have a sensory function.

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THE AFFINITIES AND EVOLUTION OF THE MAJOR TRIPLOBLASTIC PHYLA OF ANIMALS

C. J. George

Post-Graduate Department of Zoology, American College, Madurai, India

THE old group Vermes of Gegenbaur or Ehler is often said to be an incongruous medley of animals, some of them coelomate and others non-coelomate. In so far as Vermes included animals with an elongated body possessing antero-posterior orientation with an anteriorly located nervous concentration, the brain, and with nerves proceeding backwards from the brain, they represent a level of organisation well above that of the coelenterates. Again, although some of the worms are coelomate and metamerically segmented, their organisation is not so advanced as that of the arthropods which show definite determinism in the number of their segments. An earthworm or Nereis can have a varying number of segments, but not most arthropods. In their antero-posterior organisation, bilateral symmetry and the often-prevalent dorsi-ventral flattening the Vermes have a lot common between their different types of members.

The turbellarians represent the lowest rung of the ladder Vermes. They manifest an antero-posterior gradient field as well as a postero-anterior reverse field. They possess a stabilised head region at the anterior end. The posterior-most region has been stabilised as a hind extremity. Though the turbellarians are often dorsiventrally flattended, the disposition of their central nervous system indicates that they have not reached that ventro-dorsal orientation so characteristic of annelids with a ventral ganglionated nerve cord with nerves proceeding from it on either side towards the dorsal side. In many planarians there are dorsal, ventral and lateral cords with an orientation very much less stabilised than that of an annelid.

In the cestodes there is no stratification of the gradient field into metameres as in an annelid, and their head region is as indefinitely marked as in the turbellarians. But the cestodes are capable of progressively developing proglottids from the region behind their scolex and eventually severing them. The proglottids recall in some measure

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the metameres, but essentially they reflect only one aspect of metamerism, the asexual reproduction associated with metameric animals and as such they can never be equated with the morphologically and physiologically organised units, the metameres. Libbie H. Hyman, however, does not hesitate to bestow on the proglottids the status of metameres.

The annelids stand as the most advanced type of Vermes with the body stratified into metameres. In addition there is a head region and a posterior end segment, both incapable of budding off segments. They have antero-posterior, postero-anterior and ventro-dorsal orientation fields. The annelids are thus a direct evolutionary projection of the more generalised worm type—the turbellarian.

The phenomenon of growth as it takes place in the different units of Vermes reflects a characteristic evolutionary trend in that group. Theoretically an embryo of an elongated animal assumes the characteristic shape of the adult by growing cephalad as well as caudad. In some this growth takes place more or less equally in both directions. Growth in the rhabdocoelid worm Stenostomum is of this type as 'segments' are added alternately in the anterior as well as posterior halves. This growth is intercalary, the two terminal points having long ago been determined and growth affects only the intervening region. In the cestodes the posterior growth zone stops growing after the initial spurt, whereas the anterior one continues indefinitely as proglottids are budded off behind the scolex. In the annelidan trochophore intercalary growth is confined to the post-trochal region which elongates and buds off segments. After a while this growth finally stops.

In the arthropods intercalary growth is exceptional. In the proturans it takes place in the abdominal region and it is only the adding of a few segments (anamorphosis). Regeneration so characteristic of Vermes is absent in the arthropods. The total number of segments is always determined in most arthropods. The Arthropoda is near the Annelida, and Peripatus is a living example of the evolutionary bond that links the two phyla. On the other hand, in the metameric segmentation of Amphioxus and in the occurrence of solenocytes in the excretory system of that animal, there exists a genetic relationship between the Annelida and the Cephalochordata. It was natural therefore that attempts had been made in the past to connect the chordates with the annelids.

A recent tendency, however, is to support the supposed affinity of echinoderms to the chordates through the protochordates. Some have considered it advisable to create two superphyla, the Echinoderm and Annelid superphyla. In the former have been included the echinoderms, protochordates and chordates, and in the latter the annelids, arthropods and molluscs. The basis for this grouping is mainly developmental—resemblances in cleavage pattern, mode of formation of coelom and larval characters. The protagonists of this scheme of grouping are not unaware of the many exceptions which mar the validity of their contention. One definitely weak point in the scheme is that the metamerism of the chordates is assumed to have arisen independent of that in the annelids. A subtle distinction between chordate and annelidan metamerism is thus made, characterising the former type as a segmentation of the myotomes primarily. It is not, however, legitimate to ignore the segmentation of the chordate central nervous system in the embryo, for example in the chick. Nor is the close similarity between segmentation in Amphioxus and annelids easy to be brushed aside.

The creation of the two superphyla mentioned above is a logical corollary of the diphyletic theory of Grobben on phylectic evolution, which postulated the early bifurcation of the bilateral stock of animals into two, the Protostomia and the Deuterostomia. The Protostomia include those animal groups in which the mouth is formed from the blastopore or at the region where the blastopore closes. They are the worms, brachiopods, arthropods and molluscs. The Deuterostomia are those animal groups in which the anus is formed from the blastopore or at the region where the blastopore closes. They include the chaetognaths, echinoderms, hemichordates and chordates.

To those trained in anatomy and physiology this grouping of the principal phyla into two large associations appears highly illogical. The resemblance between annelids and molluscs is not certainly of the same category as that between annelids and arthropods. The common bond between annelids and molluscs is the trochophore larva and the similarity in origin of the mesoderm from two teloblast cells. These resemblances can as well exist even if they were both derived from a common ancestor. The intercalary growth which takes place in a molluscan trochophore is different from that in an annelidan trochophore. In the annelidan trochophore elongation and subsequent segmentation takes place in the post-trochal region, while in the molluscan trochophore the intercalary growth takes place without segmentation in the middle region of the larva but confined to what turns out to be its dorsal side. This elongation is almost at right angles to axis of the trochophore, but at the same time directed caudad more than cephalad. As a result, in a mollusc the visceral hump is formed, and no great elongation of the body takes place along the antero-posterior axis. The arthropods though obviously so close to the annelids show no genetic connection with the molluscs and to include the two together in a larger grouping looks fantastic. Even the recent discovery of the metameric mollusc Neopilina cannot tilt the balance very much in favour of Molluscan-arthropodan grouping, since it has yet to be shown that the assumed resemblance between that animal and a metameric one is not secondary.

Even with certain embryological resemblances such as a tripartite coelom, the chordates with a totally different organisation cannot be associated with the echinoderms. Though the echinoderm larvae are bilateral, the type of intercalary growth which takes place in them is radial, that is growth in five lateral zones. Such an intercalary growth is unique in the animal kingdom. Again the supposed affinity between Balanoglossus and the echinoderms through a superficial resemblance between their larvae, and the occurrence of a tripartite coelom is countered by the widely divergent intercalary growth in development in the two groups. Growth in Balanoglossus is intercalary in a linear direction. Moreover, the larva of Amphioxus, the only true chordate, bears no resemblance to the Tornaria or to the echinoderm larvae. The occurrence of the tripartite coelom in echinoderms on the one hand and Balanoglossus and Amphioxus on the other is to be looked upon, in the absence of collateral evidence, as only strange coincidence.

In the chordates the dorsal nerves in the head region are mixed, being composed of sensory and motor fibres, whereas the ventral ones are exclusively motor. In the body region the dorsal ones are sensory and the ventral ones motor. In the annelids and arthropods on the other hand the dorsal nerves appear to be motor and the ventral ones of mixed fibres. This reverse parallelism of the nervous plane—so it seems—has a meaning unless it is a coincidence. Is a chordate an annelid turned upside down ?

There are a few more points to be mentioned in this connection. The echinoderms and molluscs though morphologically so unlike, nevertheless show some similarity in the part their wandering cells play. In excretion and regeneration these amoebocytes are indispensable in these two groups. In the turbellarians too the amoebocytes are important in regeneration. The molluscan nervous system can very well be equated with that of some of the turbellarians in respect of the disposition of the principal cords. The echinoderm nervous system can also be compared to that of the turbellarians in its primitive nature.

The following points have been brought out in the fore-going discussion :

1. The annelids in their gradient fields and intercalary growth are a projection of the turbellarians—having acquired a new metamerism and a ventro-dorsal stabilisation of their body axis.

2. The arthropods constitute a further evolutionary step from the proto-annelids.

3. The molluscs and echinoderms possess an amoebocyte system organised for excretion and regeneration. This appears to be a heritage from the turbellarians.

4. The annelids, echinoderms and molluscs adopt diverse developmental paths to attain their respective adult condition.

5. The chordates show a reverse parallelism with the annelids and arthropods in the arrangement of their motor and sensory segmental nerves, and seem therefore basically related to the annelids.

Based on the above premises certain suggestions are hazarded here as regards the evolution and affinities of these groups of animals. The most important of them is that the echinoderms, annelids and molluscs are regarded as evolved from the turbellarians each in a different direction, primarily through a distinct type of growth. That means the three groups have come to possess each its respective type of body form and nervous system as a direct result of acquiring a different type of growth potential.

Some of the marine turbellarians creep much in the same way as the gastropods. The present day annelids compare so favourably with some of the turbellarians in their body organisation and intercalary growth that it is not surprising that some older authorities found sufficient grounds to create the composite group, the Vermes.

The Tornaria, the annelidan and molluscan trochophores and the echinoderm larvae are early-hatched out larvae and hence their mutual resemblances.

The chordate ancestors must have branched off from a proto-annelidan stock and must have moved about with their nervous-system side up. A parallel for such a movement is found in some of the crustaceans and insects moving upside down in water. It means that the ancestors of chordates must have been actively swimming creatures and not sedentary ones.

Summary

1. Arguments are advanced in support of a hypothesis that the turbellarians form a basic group from which the annelids, molluscs and echinoderms have arisen as a result of different types of growth processes.
2. The arthropods and chordates are offshoots from proto-annelidan stocks.
3. If the above points of view are conceded, the supposed direct relationship between echinoderms and chordates is untenable.

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PERMEABILITY OF A PARASITIC CILIATE (OPALINA) WITHOUT A CONTRACTILE VACUOLE

(Miss) Tripuraneni Indira,

Department of Zoology, Sri Venkateswara University, Tirupati, India

MOST of the past and recent work on permeability was done on different plant cells (such as *Chara, Nitella)* and animal cells (such as red blood cells of guinea pig, man, monkey and rabbit, eggs of sea urchin and *Fundulus* and also muscle and nerve cells) using various dyes and organic and inorganic substances. In all these cases it is suggested by the authors that the permeability is not merely by passive diffusion but it is also due to some active factor involving an expenditure of energy by the cell, to which Overton coined the term ' Adenoid activity ' and Hober ' Physiological permeability '. Recent works of Danielli, Davson and Mitchell indicate a preferential permeability by cells specially towards the metabolic substances of the cell concerned. This should be all the more true in the case of internal parasites which will have to obtain their nutrients through permeability of the body wall. Such is the case for example in several parasitic protozoa such as sporozoa and the protociliates which while experiencing wide variations in the chemical nature of their environment, yet do not possess any *organelle* either for intake or ejection of materials from and to the medium in which they live. In such cases it is to be assumed that the physiological permeability of their plasma membrane regulates such movement of material into the cell and *vice versa.* While some information is available on the sporozoa (Adcock, 1940 ; Pampapathi-Rao, 1958) little work has been done so far on parasitic protociliates which have also similar environmental problems.

Hence it is of interest to study the permeability of parasitic ciliates which have no contractile vacule or oral opening. Specially the permeability to substances which are present in the natural medium of the parasite gives us some knowledge of their selective preferences to such substances and passive diffusion of other substances and the accumulation of certain food products in the cytoplasm.

Material and Methods

Opalina, a holotrichous parasitic ciliate from the large intestine of frog was used for this study. It is found freely moving in the large intestine and also embedded in the rectal matter in innumerable numbers.

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In all cases of permeability studies, a single organism was taken with sufficient amount of host rectal fluid on a clean depression glass slide and studied under the microscope. Glass wool or transparent paper does not prevent them from their free movement.

Before starting to study the permeability of other substances, the osmotic concentration and hydrogen ion concentration of the environmental medium (rectal fluid) were found. The osmotic concentration was determined by Barger's method modified by Krogh (Prosser, 1950) based on vapour pressure. A series of concentrations of sodium chloride solutions ranging from 0.5% to 2.0% were prepared. A fraction of a drop of each concentration was taken on one side in each capillary tube of uniform diameter and on the other side an equal amount of rectal fluid was taken. Care was taken to avoid mixing up of the two liquids at the middle. The two free ends of the capillary tube are now sealed with vaseline to prevent any disturbances from outside such as entry of dust particles, free evaporation etc. The two liquid drops were measured on a centimeter graph paper and left exposed at laboratory temperature nearly 4 to 5 hours or longer if necessary and again the length was measured. If the unknown drop (rectal fluid) is more concentrated its length will increase, if it is less concentrated the drop will become smaller and it remains as it is if it is isotonic to the other liquid (NaCl). From this measurement we will get a rough estimation of the osmotic concentration. For accurate measurement another series of NaCl solutions in concentrations ranging between the two rough estimates were prepared (here it is between 1.75 to 2.0% NaCl) and the exact osmotic concentration was thus determined by matching with the rectal fluid.

To find the hydrogen ion concentration of the rectal fluid, a drop of fluid is placed on a universal indicator paper ranging between pH 2 to 10. The color developed on the indicator paper was matched with the color given on the indicator paper book. The color was found to be slightly less intense than the color at pH 8 and more intense than the color at pH 7. Hence to know the exact pH another narrow range indicator paper between pH 7 and 8 was used and the pH was found as before.

Indicator dyes cresol red and neutral red were used to find out the hydrogen ion concentration of the organism. The following procedure was adopted to determine the pH of the organism. 0.1% solutions of the indicators were prepared (Anderson, 1948). A fraction of a drop of indicator solution was added to the organism taken on a clear slide and the change in color of the indicator was observed under the microscope. The color attained within the protoplasm after penetration of the dye was noted and the corresponding pH (given in Cowdry, 1952) was taken as the pH of the organism.

In all cases the rate of permeability is measured as the time taken for the penetration of the substance into the interior of the cell. Rate of permeability to dye stuffs is easy to measure by this method due to their colour change in the cytoplasm of the cell after their entry. But in colorless substances it is difficult to perceive the time of their entry. Hence the method given by Stiles (cited by Heilbrunn, 1952) was used depending on the visible changes within the cell such as change in color of a dye in the cytoplasm after the entry of the substance used.

All dyes were used in 0.1% concentration to study the permeability. Acids, bases, and other substances whose permeability was to be measured are prepared in definite normality, in such concentration as the parasites could tolerate without toxic effect. The former two were prepared accurately by titration against base or acid as the case may be. Other substances were approximately taken in definite percentages or normal solution as the case may be. With each substance several individuals were tested to know its rate of permeability.

Results

Within the large intestine (of frog) Opalina can survive well for a considerable time even after separating the gut from the frog (nearly one day) if there is no dehydration within the gut. In dilute sucrose solution (0.1%) their survival is greater (nearly one and a half days) under aerobic conditions. Sodium chloride solution even in sufficient dilution or in isotonic solution was found to be very much toxic to the organism.

Under the microscope, Opalina appeared flat and nearly oval in shape. Myofibril bands running along the body and its multinucleate condition were very distinct. The latter condition became clear after staining with neutral red.

The osmotic concentration of the rectal fluid of frog (*i. e.* the environment of the parasite) was found to vary between 1.8 and 1.95% NaCl equivalent. The pH of the fluid was usually around 7.9 while that of the organism was also the same.

The permeability results are summarized in the following four tables (Tables 1 to 4).

TABLE I

Permeability to dye stuffs

	Indicator with molecular Wt.	Time taken to penetrate in minutes	Change in colour of the dye after penetrating into the interior of the cell	Changes in the organism due to the entry of the dye.
	Acidic dyes			
1.	Methyl orange 327.333	1′	Yellow—colourless—light pink	Two noncontractile vacuoles formed one at either end. Died 3 minutes after the formation of the vacuoles. Penetration gradual from periphery to centre.
2.	Congo red 696.658	5′	Red—light greenish blue—light pink	Before penetrating it surrounded the cell as a thin film. Penetration gradual and very slow from cortex to central plasm. After 10 minutes the organism became motionless and died.
3.	Methylene blue 319.845	15′	Light bluish green—light and dark green patches—dark green	Only after penetration of the dye, activity lessened and finally became motionless and the protoplasmic colour changed to dark green.
4.	Methyl green 458.462	Does not penetrate into the organism.		No change in the organism. Died after ten minutes.
5.	Phenol red	3′	Yellow—greenish yellow—light green	Black granules appeared here and there.
	Neutral dye			
6.	Cresol red 382.414	2′	Yellow to red	0.1% concentration found toxic to the organism soon after its penetration.
	Basic dye			
7.	Neutral red 288.775	1-2′	Red—orange—yellow	Vacuoles formed continuously at the rate of one at an interval of 5 to 10 seconds upto two minutes.

TABLE 2

Permeability to Organic acids and to Ethylene glycol

	Substance with molecular weight and concentration used	Time taken to penetrate (in minutes)	Change in colour	Change in the organism due to the addition of acid
1.	Oxalic acid 78.026 0.01 N	7′	Orange—pink—colourless	Soon after adding it shrank in size and regained after 15 minutes. Survived well upto 20 minutes. No conspicuous change in its activity.
2.	Lactic acid 90.026 0.0116 N 0.00508 N	18 sec 10 sec	Pink—colourless	With higher concentrations does not survive for more than 1½ minutes. In lower concentration survived well for a pretty long time. No marked change in the organism.
3.	Malic acid 134.048 0.01 N 0.005 N	1-2′ 2′ 10″	Light pink to yellow do	Very few survived after the entry of acid into the central plasm. Became very active as acid entered cortical region and died when it reached the central plasm.
4.	Pyruvic acid 88.032 0.01 N 0.005 N	4′	Toxic to the organism Orange—pink—Yellow	Organism shrank in size in 0.005N pyruvic acid. Gradually became immobile and ciliary beat increased rapidly and survived upto 16′. After 10′ of the entry of the acid a vacuole formed on the right posterior side.
5.	Acetic acid 60.032 0.01 ; 0.005 ; 0.0025 ; 0.00125 N	All these concentrations were toxic to the organism and it died immediately after adding the acid.		

TABLE 2 (Continued)

Permeability to Organic acids and to Ethylene glycol

Substance with molecular weight and concentration used	Time taken to penetrate (in minutes)	Change in colour	Change in the organism due to the addition of acid
6. Citric acid 192.064 0.01 N	2 to 8′	Orange—pink—colourless	Penetration gradual from the cortical region to the central plasm. Took 6′ to reach central plasm. As penetration proceeded towards the centre, organism became gradually inactive and died after 10′ *i.e.*, after 2′ after the entrance of the acid into the interior. Died with vacuolization of the cytoplasm.
7. Acotinic acid 174.048			
0.01 N	4′	Pink—colourless	Penetration gradual and movement ceased gradually. Some black stained bodies seen in the central plasm and the movement ceased in all concentrations. Did not survive more than 5 minutes.
0.005 N	2′ 10″		
0.0025 N	3′		
8. Ethylene glycol (Dihydric alcohol) 62.048			
1 N	3′		Died after 5 minutes. No change in the organism either in size or in activity.
0.5 N			Cell plasmolysed, regained size and died.
0.1 N			Penetration gradual from the periphery to the centre, and survived well for a pretty long time. After transferring into natural medium survived well.

TABLE 3

Permeability to KOH, $NH_4(OH)_2$, Urea, Uric acid and Iodine

Substance with molecular weight and concentration	Time taken to penetrate in minutes	Change in colour	Changes in the organism due to penetration of the substance
KOH			
56.104			
0.01 N	5′	Orange—yellow	Became transparent after the penetration of the alkali. Myofibrils seen distinctly. Cilia distinct and appeared arising from the cortical region. Some dark stained spots are also seen.
$NH_4(OH)_2$			
52.048			
0.01 N	Died after 11 seconds		
0.005 N	Died after 20 seconds		
0.001 N	2′		Survived after transferring into its natural medium.
Urea			
60.648			
1 N	3′		Died after 4 minutes.
0.5 N	Cell plasmolysed and died		
0.1 N	26′		In the initial stages it shrank in size and later regained its size. Survived a pretty long time. After transferring into its natural medium it survived upto 6 minutes.
Uric acid			
125.048			
0.1 N	1′		More active than before.
Iodine			
2%, 1%, 0.5%, 0.25%, 0.125%, 0.03125%, 0.078%	2′ 20″		Toxic to the organism. Dark brown stained coarse and fine particles appeared on the surface of the organism, but not in the cortical region. Became opaque but survived well.

TABLE 4

Permeability to inorganic acids

Name of the acid with molecular weight and concentration	Time taken to penetrate in minutes	Change in colour of the dye after penetrating into the interior of the cell	Change in the organism due to the entry of the acid
HCl			
36.456			
0.01 N	2′	Light orange to pink	Died after 3 minutes.
0.005 N	3′	do	Died after 5 minutes.
H_2SO_4			
98.82			
0.01 N	20″	Orange—pink—brown—yellow	Died after 1 minute.
0.005 N	2′		Died after 4 minutes.
H_3PO_4			
98.004			
1 N	30″	Pink—colourless	Died after 1′ due to vacuolization. No active movement after adding acid.
0.5 N	3′	do	
0.1 N	2′	do	Died after 5′.

Discussion

Permeability to dye stuffs :

Permeability of the basic dye neutral red is relatively more rapid than that of neutral or acidic dyes. It is perhaps because of its high lipoidal solubility. Nirenstein (1920) thought that the staining capacity of the neutral red in paramecium is due to its high lipoidal solubility. Its molecular size also is relatively less and there are many dissociable atoms. All these chemico-physical factors make the dye more permeable through passive diffusion than the others. Not only this, it has great affinity to colloidal plasm of the cell.

One exception among acidic dyes is methyl orange. Though generally acid dyes are lipoid insoluble (Overton, 1900) methyl orange is lipid soluble. Because of this lipoid solubility its penetration is rapid, though there are few polar groups in its molecular structure. This dye has got some toxic effect on the protoplasm of the cell.

The neutral dye cresol red is relatively less permeable than the basic dyes and more permeable than acidic dyes. The more the polar or methyl groups, the more rapid is the permeability of a dye (Höber, 1920). This statement seems correct with reference to this parasitic organism, exceptions being methyl green and methylene blue. The cell is impermeable to the former due to some unknown reason and only little permeable to the latter, inspite of its having relatively many methyl groups. It may be due to its want of affinity to the cell plasm or due to its readily oxidizable nature to a colorless compound in which state it is not recognizable.

Congo red penetrates less rapidly than phenol red, perhaps due to its high molecular weight (hence larger molecular size) and lesser number of polar groups.

Permeability to inorganic and organic acids :

The penetrating power of all inorganic acids was lessened with decreasing concentration of the acid, whereas the reverse was true of organic acids. In the latter, in most cases, penetration is gradual from the cortex to the central plasm, but not so in the case of the former. From these observations it is presumable that the penetration noticed in case of inorganic acids may not be through passive diffusion but through causing damage to the cell membrane. In all the three inorganic acids used the organism does not survive more than five minutes.

Among organic acids permeability to lactic acid is relatively rapid. This appears to be partly due to the lesser number of carboxylic groups, small molecular size and readily dissociable nature of the acid (weak acid). The normal activity of the organism on addition of lactic acid signifies tolerance to the acid. The color change of the indicator from orange to pink indicates the acidic nature or protoplasm after entry of the acid. The fading of the color after a time is perhaps due to the excess entry of the acid.

Oxalic acid, though possessing relatively lesser molecular size and fewer number of carboxylic groups, penetrates more slowly for the fact it is a very strong acid and hence not readily dissociable. Color change after the entry of the acid is same as in lactic acid. The activity of the organism on treatment with this acid is normal except that there is a slight shrinkage in size at the beginning.

Citric acid is relatively less permeable due to the presence of more carboxylic groups and its toxicity is not great. It is tolerated by the animal in sufficient dilutions and can perhaps be used by the parasite if available in greater dilutions. Acotinic acid exhibits the same features in its permeability and tolerance by the animal. But both these acids are capable of stopping movement and killing the organism if allowed to act for any length of time and in sufficient concentrations.

Malic acid and pyruvic acid exhibit toxicity to the organism ; the former has its toxic effect not on the cortical plasm but on the central protoplasm. As soon as the acid enters the central plasm, the organism becomes more active, but dies soon after the formation of vacuoles in the protoplasm. Pyruvic acid is relatively less toxic than the malic acid. Penetration of malic acid is relatively greater than that of pyruvic acid though the former has more carboxylic groups and molecular size. It can be presumed that malic acid penetration may be by causing damage on the cell membrane. Both the acids caused a permanent yellow color after penetrating into the organism which indicates a change into the alkaline condition.

Permeability to Urea and Uric acid :

Penetration of urea is less rapid than uric acid though it posesses a lesser number of amide groups and relatively small molecular size. In concentrations higher than 0.1 N urea is toxic to the organism. But the tolerance is very great in 0.1 N solution though penetration is very slow *i.e.* 26 minutes whereas penetration time of uric acid is one minute.

It is known from the quantitative estimation of the rectal fluid for urea and ammonia (Cullen-aeration method as modified by Van Slyke, cited in Welsh and Smith, 1952) that the percentage concentration of urea is 0.18545% in the medium. The urea used in permeability studies here is 0.156% (0.1 N). Since the concentration of urea used in this study is slightly less than what is present in the natural medium of the organism, tolerance is very great. It is presumable from these observations that Opalina is selectively impermeable to urea even in its natural medium. Shrinkage in size soon after adding urea is perhaps due to the hypertonicity of the medium.

Permeability to $NH_4\ (OH)_2$ *and KOH :*

Ammonium hydroxide is more permeable than potassium hydroxide. The organism can survive 0.01 N potassium hydroxide but not ammonium hydroxide even in greater dilusions Only in 0.001 N its survival is good. It was calculated that the amount of ammonia present in the medium was 0.06654%. But here even 0.00451% was found toxic to the organism. How it can tolerate high concentrations in its natural medium but not under experimental conditions is not known. Penetration of strong base (KOH) is greater than weak base ($NH_4\ (OH)_2$ 2) and relative tolerance is also better.

Permeability to Iodine :

Permeability was possible only in very great dilutions. The appearance of dark brown bodies after the penetration of the iodine into the interior gives us the inference that *Opalina* can well store glycogen in the central plasm. This is more evident after keeping it in sucrose solution and observing it next day by iodine test.

Summary

Opalina, a parasitic cilliate from the rectum of frog, can survive well for a pretty long time (over 24 hours) under aerobic conditions in dilute (0.1%) sucrose solution. But sodium chloride solutions even in great dilutions are toxic to the organism.

The hydrogen ion concentration of the parasite is the same as that of its natural medium, the rectal fluid. The parasite appears to be isotonic to its medium and exhibits osmotic response with volume change.

The permeability of *Opalina* to dyes follows the same general pattern as reported for other cells heretofore studied, excepting its impermeability to methyl green. In general, permeability to basic dyes is greater compared to acidic dyes, excepting to methyl orange.

Inorganic acids are toxic even in considerable dilutions. But in the case of organic acids, the molecular size, dissociability and the number of carboxylic groups in the molecule seem to determine the rate of permeability of the acid into the organism, through passive diffusion. The lactic and oxalic acids are tolerated by the organism quite well, while malic and pyruvic acids are toxic.

Penetration of urea is less rapid than uric acid. Both urea and ammonia are tolerated well in the natural medium of the parasite. But under experimental conditions ammonia is toxic even at concentrations 1/10 of the natural medium.

Tests with iodine indicate the presence of glycogen as a stored product in the organism.

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STUDIES ON THE RESPIRATION OF BARNACLES: OXYGEN UPTAKE AND METABOLIC RATE IN RELATION TO BODY SIZE IN *BALANUS AMPHITRITE COMMUNIS* (DARWIN)

P. N. GANAPATI AND D. G. V. PRASADA RAO

Department of Zoology, Andhra University, Waltair, India

IN recent years many investigations have been carried out on the respiratory metabolism in relation to body size of several marine poikilotherms from the Arctic and temperate waters. Weymouth *et al* (1944); Zeuthen (1947, 1953); Rao and Bullock (1954); Bullock (1955) and Bertalanffy (1957) carried out investigations and reviewed the work on the metabolism of several groups of marine organisms in relation to body size. However, observations on this aspect of study in barnacles are scanty. Kreps (1929a); studied the effect of salinity and pH on the O_2 consumption and CO_2 release of *Balanus crenatus* and Kreps (1929b); Barsuk and Kreps (1929) extended these investigations to *B. balanoides* and *B. crenatus*. Zeuthen (1947) studied the relative rates of oxygen consumption of different body sized barnacles of *B. (balanoides?)*. Recently Costlow and Bookhout (1958) studied respiration in relation to moulting in *B. amphitrite var. denticulata* (Broch.). From the above account it is clear that no work has been done on the respiratory metabolism of any of the barnacles occurring in the tropical waters.

Recently we have carried out a number of experiments on the respiration of *Balanus amphitrite communis* (Darwin) which is a common fouling organism at 'Station C' (Ganapati *et al*, 1958) in the Visakhapatnam Harbour. The location from where the barnacles were collected is subjected to very wide fluctuations of hydrographical conditions. The salinity ranges from 5‰ during October to 34‰ during the summer months. Owing to heavy pollution consequent on the discharge of the town's sewage, very near this station, the dissolved oxygen content in the water is low varying, from 0.1 ml.O_2/L. to 3.3 ml. O_2/L. The present studies therefore, are not only of fundamental interest in understanding the physiological adaptations of these animals to such widely fluctuating environmental conditions, but are also of considerable interest in under-

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standing problems associated with fouling. In the present paper we have given an account of the metabolism in relation to the body size of *B. amphitrite communis.*

Material and Methods

Respiratory measurements were made using the standard Winkler's method (Welsh and Smith, 1953) at a constant temperature of 25.5 °C. and a salinity of 30‰. The respiratory chambers were designed after Erichsen-Jones (1947a). Wide mouthed pyrex bottles of 270 c.c. capacity were used as respiratory chambers. The temperature of the bath varied only ± 0.5 °C. throughout the experimental period. Whatman 42-filtered sea water of a fairly high oxygen content was used. The pH of the sea water used during different experiments did not vary very much. The experiments were run for a period of 6 hours and the average hourly O_2 consumption was calculated. The wet weights of the animals were determined after the experimentation to the nearest ten milligrams. All the animals were cleaned and acclimatized to the experimental conditions for 24 hours. In all, ten series of experiments were conducted involving 100 animals of 12 different size groups. In Fig. 1 and 2 the average oxygen uptake and the metabolic rate respectively are plotted against the body weight on double-log graph.

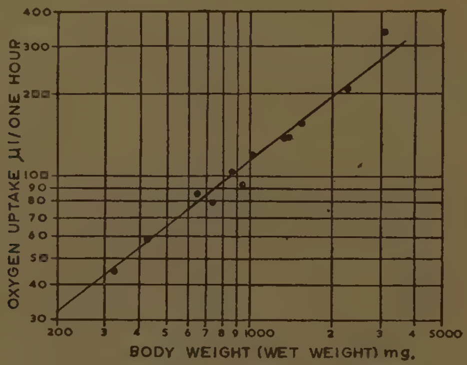

FIG. I. DOUBLE LOG PLOT OF AVERAGE OXYGEN UPTAKE AGAINST BODY WEIGHT

Results

The oxygen uptake of *B. amphitrite communis* varied from 45-300 µl O_2/hr. and the metabolic rate from 140-94 µl O_2/gr./hr. depending on the size of the animal. As shown in Fig. I the oxygen uptake of these animals

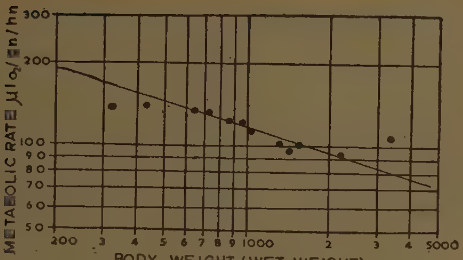

FIG. 2. DOUBLE LOG PLOT OF AVERAGE METABOLIC RATE AGAINST BODY WEIGHT

increased with increasing body weight. The increase in the oxygen uptake showed an exponential relationship with the body weight. The dependence of the metabolism of an animal on body size can be expressed in the form of an equation $Y=aX^b$ (Zeuthen 1953), where 'Y' is the oxygen uptake in unit time, 'X' the body weight and 'a' and 'b' are constants. Using this equation we calculated 'b' by statistical method and obtained a value of 0.827.

The metabolic rate or the rate of oxygen uptake per unit body weight in unit time as shown in Fig. II declined with increase in body size, and the line of regression of the logarithmic plot is negative. We have been able to get only a single animal, each, for observations on the extreme size groups and the scattering of the results at the extremities may be due to the inadequacy of the number of observations. The slope of the line of regression of the metabolic rate in Fig. II is characteristically similar to that observed for various other marine crustacea. Also, the value of 'b' calculated by us from the above equation is very near to the value 0.80 obtained for other marine crustacea. The slight deviations of the results of our present study from those of others made elsewhere might be due to difference in the range of size groups of the animals used and their individual physiological variations.

Discussion

The metabolic rate or the weight specific O_2 consumption in *B. amphitrite communis* (Darwin) showed the characteristic decrease with increasing body size as was observed by Zeuthen (1947) in *B. (balanoides ?)*. The line of regression of metabolic rate was also approximately similar in character to that obtained by him for other crustacea of similar size ranges. Making an interspecific comparison of the 'b' values obtained by other workers for various marine crustacea, Zeuthen (1953) also stated that the values of 'b' obtained by him in animals with body nitrogen less than 1 mg. to be 0.95 and for animals above this weight the value of 'b' was smaller *i.e.* about 0.80 for marine crustacea. The animals studied by him ranged from 0.4 μg. to 4 grms. in body weight. The body weight studied by us ranged from 0.3 to 3.0 grms. and the value of 'b' of our experiments (0.827) agrees very closely with his results. However, we could not make any observations at present on animals above and below the range mentioned. The temperature relationship of 'b' values for these animals will be discussed elsewhere.

Summary

1. The oxygen consumption of different size groups of *Balanus amphitrite communis* (Darwin) under constant temperature and salinity increased with increase in body weight.
2. The metabolic rate showed a decrease with increasing body weight.
3. Using the equation $Y = aX^b$, 'b' was calculated by statistical method and a value of 0.827 was obtained.

Acknowledgements

We are thankful to Dr. S. Dutt for help in the statistical presentation of the data.

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OXYGEN CONSUMPTION AND OXIDATION OF VARIOUS METABOLITES BY THE PIGEON ADIPOSE TISSUE, *in vitro*

J. C. George and J. Eapen

Laboratories of Animal Physiology and Histochemistry, Department of Zoology, M. S. University of Baroda, Baroda, India.

Adipose tissue has long been regarded as a storage form of connective tissue with very little metabolic activity of its own. However, it is being realised more and more at present, that this belief is illfounded, and sufficient evidence has already accumulated to show that it is metabolically active and is even on a par with other well-known metabolically active tissues. The *in vitro* oxygen consumption by the adipose tissue of the rat was studied by Mirski (1942) and of the ground-squirrel by Hook and Barron (1941). But so far as we are aware, there is no comparable work on the avian adipose tissue. In the present study, the oxygen consumption by the pigeon adipose tissue (in Ringer phosphate and Krebs-Ringer bicarbonate, without added substrates and Co-factors) and the oxidation of various metabolites by the same tissue in the absence of Co-factors, have been studied, *in vitro*.

Material and Methods

The material used was the visceral adipose tissue, covering the coils of the intestine, of the pigeon *(Columba livia)*. After decapitation of the pigeon, the tissue was removed and transferred to a cold watch glass placed on cracked ice and was then cut into small slices with a pair of clean scissors.

The oxygen consumption by the adipose tissue in the absence of added substrates and Co-factors was studied as follows. 100 ml of 0.154 M NaCl, 4 ml of 0.154 M KCl, 1 ml of 0.319 M $MgSO_4$ and 30 ml of phosphate buffer of pH 7.4 were mixed and stored at 4°C. The final pH of the mixture was 7.4. 3 ml of this was taken as the incubation medium, in each Warburg flask having centre well and one side arm. In addition to the above Ringer phosphate medium, Krebs-Ringer bicarbonate medium was also tried. This medium is made up of the following : 100 ml of 0.154 M NaCl, 21 ml of 0.154 M $NaHCO_3$, 4 ml of 0.154 M KCl, 1 ml of

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0.154 M KH_2PO_4, 3 ml of 0.11 M $CaCl_2$ and 1 ml of 0.154 M $MgSO_4$ (Hagen *et al*, 1959). In each set of experiments the adipose tissue from the same pigeon was used for both the media. This was done with a view to compare the amount of oxygen consumed in these two media by the adipose tissue slices. The flasks were kept cool till they were transferred to the bath (37°C), by keeping on cracked ice. The slices of adipose tissue were weighed rapidly and transferred into the flasks by means of a pair of cold, clean, dry forceps. 100-300 mg of the tissue was taken in each case. The centre well contained 0.2 ml of 10% KOH with filter paper, thus making up a total fluid volume of 3.2 ml. Incubation was carried out for 1 hour at 37°C with air as the gas phase. After an equilibration period of 10 min., the readings were taken at regular intervals for 1 hour. The results are expressed as µl O_2 consumed/mg protein/hour and µl O_2 consumed/100 mg wet tissue/hour. Protein content of the adipose tissue was determined by the micro-Kjeldahl steam distillation method for total proteins (Hawk *et al*, 1954). For the determination of protein 20-40 mg fresh tissue was taken.

For the study of oxidation of various metabolites (glucose, glycogen, sodium succinate, pyruvate, lactate, citrate, acetate, glycerophosphate) the same procedure was followed. The volume of Ringer phosphate was reduced from 3 ml to 2.5 ml and to this 0.5 ml of 0.1 M solution of the metabolite was added except in the case of glycogen and glycerophosphate where the concentration of the solution used was 0.1% instead of 0.1 M. 0.2 ml of 10% KOH was kept in the centre well with filter paper. Control flask contained 3 ml of Ringer phosphate solution in the main chamber and 0.2 ml of 10% KOH in the centre well with filter paper. Both sample and control flasks each contained in addition 100-300 mg of sliced tissue which was obtained from a single site. The values given for oxidation of the various metabolites are the figures obtained after substracting the values of oxygen consumed by the tissue in the control flask from that of the sample.

In the presence of added substrates like glucose and acetate synthesis of fatty acids may also take place. To investigate this aspect the following procedure was adopted (Ball *et al*, 1959). 100-300 mg of adipose tissue was placed in a Warburg flask with 3 ml of Ringer phosphate containing 3 mg/ml glucose or acetate, without any KOH in the centre well for the absorption of CO_2, with air as the gas phase. The flasks were kept cool till they were mounted and transferred to the bath (37°C). After an equilibration period of 10 min., the readings were taken after 1 hour. If the oxygen consumption of the tissue equals its carbon dioxide output, then no pressure change should be observed. If on the other hand, carbon dioxide output exceeds the oxygen consumption, then a positive pressure should develop which could be measured. If the reverse is the case (R. Q. less than 1), then a negative pressure should develop which could be also recorded. Ball *et al* (1959) applied this method to a study of fat synthesis by adipose tissue of the rat.

Results and Discussion

Table 1 presents values of the oxygen consumption of the adipose tissue, in the absence of added substrates and Co-factors, in the two media, Ringer phosphate and Krebs-Ringer bicarbonate.

TABLE 1

Experiment	Medium			
	Ringer phosphate		Krebs-Ringer bicarbonate	
	µl O_2/100 mg/h.	µl O_2/mg protein/h.	µl O_2/100 mg/h.	µl O_2/mg protein/h.
1	11.64	4.70	12.56	5.07
2	11.79	4.76	8.53	3.44
3	8.41	3.39	5.67	2.29
4	7.57	3.06	4.98	2.01
5	13.56	5.47	12.13	4.90
6	13.68	5.52	—	—
7	20.53	8.29	—	—
Mean	12.45	5.03	8.77	3.54

From the results obtained it is evident that Ringer phosphate is a better medium than Krebs-Ringer bicarbonate. According to Hagen *et al* (1959) Krebs-Ringer bicarbonate medium is the most satisfatory of all the media tried by them, for the rat adipose tissue.

Of the various metabolites added, succinate was found to be oxidized to the maximum and the oxidation of pyruvate was to the minimum. Citrate also gave a very low value (Table 2).

TABLE 2

Substance*	μl O_2/200 mg/h.		
	Sample	Control	Difference
glucose	13.58	9.86	3.72
glycogen	20.15	14.58	5.57
succinate	36.18	11.14	25.04
pyruvate	12.58	10.83	1.75
citrate	7.75	5.89	1.86
acetate	11.08	7.65	3.43
glycerophosphate	22.0	15.42	6.58
lactate	14.12	7.76	6.36

*The values presented in each instance is the average of 5 experiments.

Mirski (1942) reported that, addition of pyruvate and glucose increased the oxygen uptake of the adipose tissue of the rat, while lactate and glycogen had no effect. In our present studies all the metabolites tried increased the oxygen consumption to a lesser or greater extent.

The presence of glucose or acetate did not bring about fatty acid synthesis as was seen in the experiments conducted to assess the possibility of fatty acid synthesis. The pressure inside the manometers remained slightly negative indicating oxygen consumption. The adipose tissue in such cases maintains an R. Q. below 1. Ball *et al* (1959) also found that fatty acid synthesis did not take place in the presence of glucose alone. When insulin was also present in the medium, fatty acid synthesis occurred (Ball *et al*, 1959).

The protein content of the fresh adipose tissue of the pigeon ranged from 1.446 mg/100 mg to 4.666 mg/100 mg, with an average of 2.478 mg/100 mg (Table 3).

TABLE 3

Experiment	mg protein / 100 mg tissue
1	3.243
2	3.115
3	1.816
4	1.577
5	2.326
6	1.803
7	1.953
8	1.792
9	2.253
10	1.446
11	4.666
12	3.745
Mean	2.478

The wide variation in the protein content of the adipose tissue is mainly due to the unequal amounts of fat present in this tissue in the different pigeons.

Summary

The oxygen consumption of the adipose tissue without added substrates and Co-factors was studied, in Ringer phosphate and Krebs-Ringer bicarbonate media, by the Warburg manometric method. The values were on the average 12.45 µl O_2/100 mg/hour and 8.77 µl O_2/100 mg/hour respectively, in Ringer phosphate and Krebs-Ringer bicarbonate. Ringer phosphate was found to be better than Krebs-Ringer bicarbonate for the pigeon adipose tissue. The oxidation of various metabolites in the absence of added Co-factors was also studied. Succinate was found to be oxidized the most and pyruvate the least, with mean values of 25.04 µl O_2/200 mg tissue/hour and 1.75 µl O_2/200 mg tissue/hour respectively. The average protein content of the fresh pigeon adipose tissue was 2.478 mg/100 mg tissue.

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THE PRESERVATION OF BOVINE SEMEN AT ROOM TEMPERATURES

UDHISHTRA DEVA SHARMA

Post-Graduate College of Animal Sciences, Izatnagar, U. P., India.

SOME of the problems that largely engaged the attention of workers in the field of artificial insemination (A. I.) since the pioneering efforts of the Russian workers headed by Ivanov were concerned in finding a superior dilutor that would extend and preserve the life and fertilizing capacity of ejaculated spermatozoa over long periods. Beginning with the studies of Phillips (1939) work upto 1957 has been reviewed by Salisbury (1957) and later in detail by Sharma (1959a). The diluents developed, so far, invariably contained a biological fluid like egg yolk or milk and preservation depended upon some kind of refrigeration. No satisfactory medium of precisely known chemical ingredients has yet been developed, nor any detailed investigations undertaken to study the possibility of preserving the life and function of the spermatozoa without the use of refrigeration. This is all the more remarkable as it is common knowledge that spermatozoa of various species are capable of retaining motility and fertility for long periods in male and female reproductive tracts. Thus in the male, the maximum motile life of sperm in isolated or ligated epididymis has been reported to be about 42 days in the rat (Moore, 1928 ; White, 1930), ~~60 days in the mouse~~ (Benoit, 1925) 65-70 days in the guinea pig (Moore, 1928 ; Young, 1929), 60 days in the rabbit (Hammond and Asdell, 1926) and over 60 days in the bull (Kirillov and Morozov, 1936). The fertilizing capacity, however, did not last so long. In the female, the case of the bat is unique. Reports of Hartman (1933) and Wimsatt (1942 ; 1944) have shown that sperm remained capable of fertilizing eggs in spring after being in the female genital tract for several (5-8) months. In the fowl, Crew (1926) reported that the fertilizing capacity was retained by the sperm upto 32 days. In lower reptiles and aquatic animals the recorded periods for which spermatozoa retain fertilizing ability run into several months (Purser, 1937 ; Rahn, 1940) and even years (Hilderbrand, 1929). It would thus seem that a continuous supply of nutrients

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and removal of waste products which is accomplished through circulation in the body, may be of greater significance in sperm survival than temperature. A study of factors which enable sperms to survive and retain fertility at higher temperatures in the mammalian reproductive tracts should provide a basis for their preservation at room temperatures. Using the permeable membrane technique developed by VanDemark and Coutourier (1956) and Walton and Dott (1956) a detailed study of this aspect of the problem was undertaken and the results are reported in the present paper. Broadly, the investigations had a three fold purpose.

1. Determination of individual nutrient requirements of sperms for survival and fertility.

2. Development of a satisfactory medium of known chemical composition consistent with good survival and fertility during storage.

3. Preservation of bovine semen at room temperatures with a view to eliminating refrigeration in routine, artificial insemination work.

Material and Methods

Semen: Bovine semen from one to two hours old and of reasonable quality collected by artificial vagina was used in the experiments reported in this paper.

Sperm concentration: The number of sperms per ml. of semen was determined photo-electrically according to the method of Salisbury *et al*, (1943).

Motility: The motility of the sperm was observed microscropically in diluted semen at 100°F. The percentages of moving sperms were determined in several fields and recorded to the nearest 5%. The rate of motility was based on arbitrary scale in which 4.0 represented maximum motility and 0.0 represented no observable activity. Half grades were frequently given to describe grades of motility falling between the full grades. A rating of 0.1 and 1% was given when even sliget sperm activity was observed.

pH: The pH determinations were made with a standardized model G Beckman pH meter.

Temperature: Most of the experimental work was done at ordinary uncontrolled room temperature (76° ± 16°F), except when a refrigerator was employed to store semen at 40°– 42°F. Constant temperature rooms were employed for storing semen where effects of precise temperature were under investigation. Records of storage temperatures were maintained with a thermograph, where possible.

Preparation of media :—Reagent grade chemicals were used in the preparation of solutions.

Osmotic pressure :—Osmotic pressure (OP) was measured in milliosmoles (MO) with a standardized Fiske osmometer. A reading of 290 MO was considered isosmotic with semen. Calculated amounts of chemicals were added to glass distilled water and resulting solutions were checked for osmotic pressure. At times the solutions were either discarded or had to be adjusted to approximately 290 MO by the addition of chemicals or water but ordinarily calculated weights of chemicals yielded isosmotic solutions.

Preservation techniques :—*Dialysis technique*—Provision of a continuous supply of nutrients and removal of waste products were accomplished by using a modification of the dialysis technique described by VanDemark and Coutourier (1956), illustrated in Figs. 1 and 2. A flask of diluent placed at a higher position was connected with a U tube by means of a flexible plastic tube to act as a siphon and furnish a stream of fluid through the U tube. The flow could be regulated with a needle screw valve at the exit and the stream could be interrupted at will by clamping the flexible siphon. Attempts were made to control the flow of diluents at a uniform rate with a 'Sigmamotor', without much success. Raw semen was stored in a narrow cellophane visking casing (size 8/32) with provision at the top for the removal of samples for examination. The cellophane sac was then placed in the inner arm of the U tube while the diluent bathed the outer surface of the sac. The exchange of materials took place across the membrane by diffusion without the sperm coming in direct contact with the medium. The flow of fluid through the U tube varied from 100-150 ml. per day but was nearly uniform for all treatments in any one experiment.

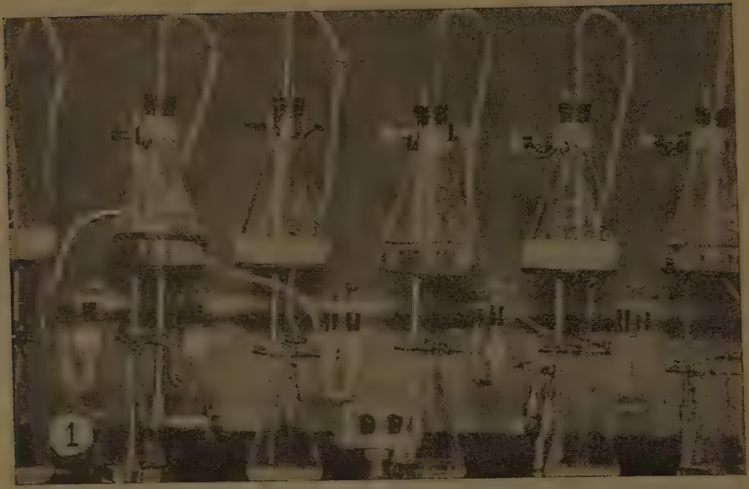

Figure 1. Units of flow dialysis

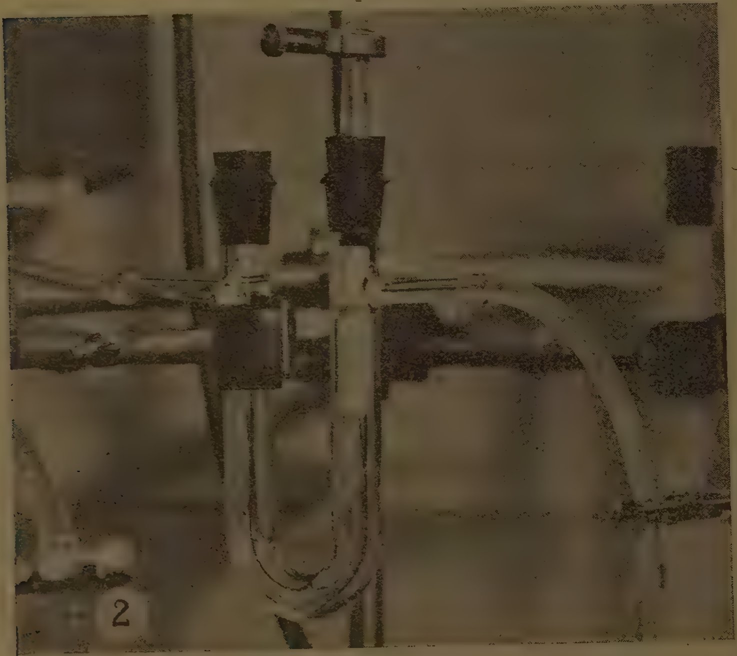

Figure 2. A close up of the U-tube.

RESULTS AND DISCUSSION

Exploratory experiments

A series of experiments were carried out to determine the essential requirements of sperm for '*in vitro*' survival. Different techniques of preservation were also tried. Results are briefly summarized below :

1. Using the flow dialysis technique the survival times of sperm kept in 0, 5, 10, 15 and 20 per cent sucrose solutions having, 0, 143, 308, 545 and 783 osmotic pressure (MO) were compared with that of neat semen. None of the concentrations used provided survival comparable to untreated neat semen (Control). Survival decreased progressively with increase of sucrose concentrations above 10% which is taken as isosmotic. At isosmotic level, survival time was 8 hours as compared to 14 hours of controls.

2. Isosmotic NaCl, $CaCl_2$, $MgSO_4$, KH_2PO_4, $NaHCO_3$, Calcium free Krebs Ringer, Krebs Ringer (KR), Krebs Ringer Phosphate (KRP) and Krebs Ringer Bicarbonate (KRB) solutions at pH 7.0 were used for storing semen in flow dialysis (FD) and in small test tubes (TT) in suspensions containing 1 part semen and 10 parts diluent (1 : 10). The survival was uniformly better in test tubes than in flow dialysis where NaCl, $NaHCO_3$ and modifications of Krebs Ringer solutions gave better sperm survival. The highest was in KRB (27 hours for FD and 37 hours in TT as compared to control of 31 hours). Phosphate had the lowest survival time (11 hours for FD and 14 hours for TT) and appeared to depress motility.

3. NaCl, $NaHCO_3$, KR, KRB and KRP solutions were selected and average survival times of sperm in these five solutions containing 0.10% and 0.30% glucose and none with the two techniques (FD and TT) were determined. Greatest improvement in survival time was found in KRB solution (63 hours in FD, 47 hours in TT as opposed to 38 hours for control neat semen)

4. Due to the depressing action of phosphate, this was omitted from the trials and in the experiment now undertaken, the effect of 5 levels of KCl, $CaCl_2$ and $MgSO_4$ in NaCl solution (15 modifications of Krebs Ringer solution) and their effects on sperm survival were studied with FD and TT techniques using a 3-dimensional central composite design of experiment as suggested by Box and Wilson (1951) and Box (1954). KCl, $CaCl_2$ and $MgSO_4$ levels varied from 0-7.34, 0-5.50

and 0-1.84 ml. of isosmotic solutions respectively in 100 ml. of the final solution in which the final volume was made up with isosmotic NaCl solution. The KH_2PO_4 component of Krebs Ringer solution was omitted. TT was found to be slightly superior to FD. Analysis of variance of the data gathered showed no marked differences between solutions, though between ejaculates, variance was highly significant.

5. The above experiment was repeated adding isosmotic glucose solution to give a final 0.30% concentration. Results were similar to those in the previous experiment.

6. Next 0.208% $NaHCO_3$ was added to the 15 solutions of experiment 4 above and the whole experiment repeated. Best results with both techniques were obtained with solutions that had no $CaCl_2$ and $MgSO_4$. Here the respective survival times in hours in the two (FD and TT) techniques were 27 and 42 and 28 and 41; total motile sperm rates too were high. The presence of KCl appeared to have a beneficial effect on sperm in FD technique. The survival time for untreated raw semen was 32 hours. Statistical analysis of the data showed significant variations due to solutions used.

7. Experiment 4 was repeated using both $NaHCO_2$ and 0.30% glucose as added constituents of solutions. Unlike the previous results, in this experiment FD technique was found by far superior to the TT technique and Krebs Ringer's solutions containing to $MgSO_4$, and $CaCl_2$ salts gave the best results (81 and 78 hours respectively as compared to 34 hours of neat semen). With FD, statistical analysis brought out highly significant variation due to solutions. Plotting the average responses in a three dimensional figure indicated a trend showing a favourable effect on motility and livability due to the addition of KCl and an unfavourable effect on the addition of $CaCl_2$ or $MgSO_4$ with the flow dialysis technique.

8. In the experiments reported so far, the solutions were adjusted to pH 7.00 by the addition of HCl. This is laborious. Since bovine sperm are reported to have a wide range of tolerance between pH 6.0 and 8.0 (Blackshaw and Emmens, 1951) and the pH values of unadjusted Krebs Ringer Bicarbonate solutions were about 8.0 it was thought that the adjustment to pH 7.0 may be unnecessary. The results at pH 8.0 were in general agreement with those obtained in the previous experiment.

9. In this experiment, the only addition made from that of the previous experiment was to gas the solutions for 10-15 minutes with CO_2

gas. This had the effect of lowering the pH of the solutions to about 6.1 to 6.2 due to the formation of carbonic acid. The pH lowered by CO_2 gradually rises due to the decomposition of carbonic acid and loss of CO_2 from the solution. Once again with FD technique the highest survival 117 hours was observed in the solutions containing no $CaCl_2$ and lowest (72 hours) in those in which KCl was absent. FD was superior to TT. Statistical analysis showed that the between ejaculates and between solutions, variations were highly significant in both the treatments.

10. In this experiment everything remained as before (Expt. 9) except that there was an addition of 0.30% sulfanilamide. Addition of sulfanilamide produced marked improvement in livability and motility of semen in the solutions. Flow dialysis technique was on the whole superior to the TT technique. The highest response (117 hours) was once again found in solutions containing no $CaCl_2$ and the lowest (39 hours as compared to 21 hours of neat semen control) in solutions containing no KCl. Statistical analysis gave results similar, in general, to those in Expt. 9.

11. Experiment was also carried out using solutions of sodium citrate, $NaHCO_3$ and NaCl containing the other constituents, but no $CaCl_2$ and $MgSO_4$. The solutions were gassed for 10-15 minutes prior use. Results are given below in Table-1.

TABLE 1

Response of sperm to NaCl, $NaHCO_3$ and sodium citrate solutions containing KCl, 0.30% glucose and 0.30% sulfanilamide and initially gassed with CO_2 with two storage techniques

(average of four ejaculates)

	Solution* average OP (MO)	Ml KCl (1.15%)	Ml NaCl (0.90%)	Ml $NaHCO_3$ (1.30%)	Ml $Na_3C_6H_5O_7$ 2 H_2O (2.90%)	Total survival (hrs.)		Total motile Sperm rate			
						FD	TT	FD	TT	FD	TT
1	296	2.68	70.32	16	—	108	48	275	209	22	14
2	301	2.68	—	16	70.32	129	57	323	213	24	14
3	306	2.68	—	86.32	—	102	57	233	202	18	13

* 6 ml. glucose (5.0%), 0.3 gm. sulfanilamide and 5 ml. water were added to each solution to furnish the desired concentration of these agents. The solutions were then gassed for 10-15 min. with CO_2.

As in previous studies, FD gave better results than TT and further, solutions with citrate as one of the ingredients gave the best results.

The analysis of variance for per cent motile sperm with the dialysis technique and in 1:10 dilutions in test tubes was carried out to study the manner in which the individual salts and their concentrations affected the response of the sperm. Only the conclusions are summarised below:

The overall effect of the addition of KCl and $MgSO_4$ was beneficial but $CaCl_2$ exerted a harmful influence. The effects of $CaCl_2$ and $MgSO_4$ were not significant. There was no significant interaction between KCl, $CaCl_2$ and $MgSO_4$. The quadratic mean square was significant and the quadratic regression coefficients were all negative, indicating the existence of an optimum which was found to be at 3.22, 1.84 and 0.73 ml. per 100 ml. of isosmotic solutions of KCl, $CaCl_2$ and $MgSO_4$ respectively. However, since the mean square for the lack of fit also was significant, the general quadratic in three variables was not capable of representing the data within the limits set by the experimental error, casting some doubt on the calculated location and value of the optimum. For the TT technique, the analysis showed that the quadratic mean square was not significant, indicating that there was no optimum in the vicinity of the region explored.

Conclusions based on results of experiments 1-11

On a general and detailed appraisal of the results obtained from experiments 1-to 11, it was clear that among the various solutions and ingredients tried, the two below detailed solutions of the specified composition could be depended upon to maintain uniformly good motility during storage in the presence of 0.30% sulfanilamide and when gassed with CO_2.

Soln. No.	O. P. (M.O)	Ml. KCl (1.15%)	Ml.NaCl (0.90%).	Ml. $NaHCO_3$ (1.30%).	Ml. $Na_3C_6H_5O_7.2H_2O$	Ml. $MgSO_4.7H_2O$ (3.82%).	Ml. Glucose 5.0%	Ml. water
1	293	2.68	69.65	16	—	0.67	6	5
2	301	2.68	—	16	70.32	—	6	5

Role of CO_2 in the preservation of Sperm :

Further experiments (not reported here) revealed that CO_2 had a beneficial effect on the sperm. It is suggested that the CO_2 affects sperm survival in any, or all of the following ways :

1. CO_2 in the presence of $NaHCO_3$ forms a more efficient buffer system for *in vitro* preservation of bovine semen at room temperatures.

2. CO_2 has a depressant or narcotic action on the sperm, inducing reversible immotility and reduction of metabolic processes resulting in con-

servation of energy and prolongation of life of the sperm during storage. The narcotic influence of CO_2 has been reported in cells from lower forms of life (Becker, 1936) and there is evidence that the same may be true for sperm (Shettles, 1939, 1940). A suggestion of some beneficial effect of CO_2 on bovine sperm may be detected in the earlier experiments of VanDemark and Couturier (1955). Our experiments have indicated a reversible narcotic effective of CO_2 on bovine sperm. In media gassed with CO_2 certain immotile sperm would gain movement and develop vigorous motility after being on a warm stage for some time. This was particularly so when semen was examined just after mixing with media freshly gassed with CO_2. CO_2 is capable of influencing the cells even if it passes into them from $NaHCO_3$ (Jacobs, 1922).

3. CO_2 is utilized by the sperm cells either directly or indirectly. Several biochemical reactions concerning CO_2 fixation such as the formation of oxalacetate from phosphoenol pyruvate (Utter and Kurahashi, 1954) formation of L-malate from pyruvate (Ochoa, 1952) and the formation of D-isocitrate from α-ketoglutarate (Ochoa, 1948) have been demonstrated. The beneficial effect of CO_2 on sperm might be mediated by the enzymes concerned in these reactions, if present. For example, the CO_2 might promote carboxylation of phosphoenol pyruvate to oxalacetate and thus decrease lactic acid accumulation to sub-toxic levels. In addition, increase in CO_2 tension would effectively decrease the observed oxidative metabolism by increasing the rate of reductive carboxylation as cited above. This area of knowledge in sperm physiology and its exploitation for the preservation of semen remains to be explored.

Action of CO_2 gas on the motility of bovine sperm :

1. Narcotic effect

Direct observations on diluted semen samples coming from 12 different ejaculates kept in special chambers and subjected to CO_2 gassing were made. In every instance, the motility of sperm decreased markedly in an atmosphere of CO_2. In some instances, the motility ceased completely in a matter of a few minutes. This was reversed when CO_2 was replaced by air, oxygen or nitrogen. In contact with nitrogen, the sperm maintained normal motility. Observations were repeated in a variety of diluents with like results. The pH of diluents never fell below 6.0 after treatment with CO_2. It is felt that the cessation of motility after exposure to CO_2 was due neither to anoxia nor to high acidity, but to some special action of CO_2 on sperm.

2. Effect on motility

The possibility of utilizing the depressant action of CO_2 on sperm motility for preservation of sperm at room temperatures was then investigated. For this purpose, four ejaculates were stored in Thunberg tubes under air (at atmospheric pressure), vacuum (about 2-3 lbs. atmospheric pressure per square inch), nitrogen and CO_2 in 1 : 10 dilutions in a new diluent developed on the basis of the findings detailed in part in this paper and called Illini Variable Temperature diluent or IVT for short (details given elsewhere, see below) with or without egg yolk. The room temperatures which were recorded during the experiment varied from 70° to 88°F. Results (given in Table 2) showed that in both the diluents, livability and motility were maintained much better under CO_2 than under other treatments which were about the same in their ability to support motility although nitrogen and vacuum seemed to have a very slight beneficial effect over storage under air. Table 2.

TABLE 2

Motility of sperm during storage in 1 : 10 Dilution at room temperature (79 ± 9°F) in Thunberg Tubes under air, vacuum, nitrogen and CO_2*, in IVT diluent with and without yolk*

(average of 4 ejaculates)

Days of storage	I. V. T. diluent without yolk							
	Air		Vacuum		Nitrogen		CO_2	
	%M	Rate	%M	Rate	%M	Rate	%M	Rate
0	58	3.0	58	3.0	58	3.0	58	3.0
1	45	2.3	49	2.4	48	2.4	53	2.8
2	33	1.6	36	1.5	36	1.8	45	2.4
3	24	1.1	28	1.1	24	1.4	40	2.0
4	11	0.8	15	0.8	15	0.9	34	1.8
5	6	0.4	6	0.4	9	0.5	28	1.5
6	3	0.3	3	0.1	5	0.3	18	1.1
7	1	0.1	1	0.1	1	0.1	14	0.9
8	0	0	0	0	0	0	10	0.6
9	0	0	0	0	0	0	5	0.3
10	0	0	0	0	0	0	3	0.3

Days of storage	I. V. T. diluent with 10% yolk							
	Air		Vacuum		Nitrogen		CO_2	
	%M	Rate	%M	Rate	%M	Rate	%M	Rate
0	58	3.0	58	3.0	58	3.0	58	3.0
1	46	2.3	46	2.4	49	2.4	51	2.8
2	36	1.8	40	1.8	40	1.9	49	2.5
3	23	1.3	28	1.3	28	1.3	43	2.3
4	16	0.9	16	0.9	16	0.9	36	1.9
5	4	0.4	8	0.5	4	0.4	28	1.6
6	0	0	0	0	0	0	20	1.5
7	0	0	0	0	0	0	15	1.0
8	0	0	0	0	0	0	13	0.8
9	0	0	0	0	0	0	2	0.2
10	0	0	0	0	0	0	0	0

In some instances, livability was poorer in IVT diluent with yolk due presumably to bacterial multiplication because of egg yolk. The beneficial narcotic or depressant effect of CO_2 on sperm stands confirmed through the results just reported. The full mechanism underlying this narcotic effect of CO_2 on sperm has not been yet investigated. It is possible that this effect is due in part to the ability of the CO_2 to penetrate the cells and enter into reversible combinations with cellular proteins as has been shown in studies with *Tradescantia verginica* and *Paramecium caudatum* (Becker, 1936).

The existence of concentrations of CO_2 having a beneficial narcotic effect and a toxic effect on sperm cannot be ruled out. In fact, Shettle's (1939, 1940) reports indicate that CO_2 can cause reversible as well as irreversible immotility in human sperm with varying concentrations and repeated exposures. Our studies showed no immediate toxic effect of CO_2 on bovine sperm. It is possible that toxic levels of CO_2 were not attained under the conditions of these investigations, or that other substances in the diluting media exerted a protective action against any injurious effect of CO_2. Perhaps, the success of the ampouling technique (Sharma, 1957, 1959b) and beneficial effect of gassing the medium with CO_2 might be due to a chance realization of an optimum, or near optimum concentration of CO_2 for the preservation of bovine sperm at room temperatures. However, the exact optimum and toxic levels of CO_2 for bovine sperm remain to be determined.

The Illini Variable Temperature (IVT) Diluent:

The Illini Variable Temperature (IVT) Diluent evolved ultimately for preserving bovine semen at room temperatures had the following composition (The figures are per litre of distilled water).

1.	Sodium citrate dihydrate	20 01	gms.
2.	Sodium bicarbonate	2.01	,,
3.	Potassium chloride	0.40	,,
4.	Glucose	3.00	,,
5.	Sulfanilamide	3.00	,,
6.	Penicillin	1×10^6	IU.
7.	Streptomycin	1.00	gms.

Gassing by CO_2 for about 10-15 minutes (pH 6.2-6.3) and mixed with 10% egg yolk.

Below are described the results of tests carried out with IVT.

Tests with IVT Diluent:

(a) **Effect of 5 levels of egg-yolk, five cell concentrations and 5 temperatures on response of sperm during storage (in ampoules).**

The levels of egg yolk tried were 0, 5, 10, 15 and 20% yolk, the cell concentrations employed were 2, 12, 22, 32, 42, $\times 10^6$ cells per ml., and temperatures were 40, 55, 70, 85 and 100°F.

Results for five different ejeaculates are summarized in Table 3.

TABLE 3

Response of sperm stored in 3.4 ml. capacity ampoules at 5 levels of egg yolk, 5 cell concentrations and 5 temperatures

(average of 5 ejaculates)

Treatment	% yolk ml.	Cell Conc. $\times 10^6$/ml.	Storage Temp. (°F)	Total survival (days)	Total motile sperm %	Rate
1	5	12	55	7	205	9.8
2	15	12	55	7	204	9.8
3	5	32	55	7	208	10.0
4	15	32	55	7	206	9.9
5	5	12	85	4	152	7.3
6	15	12	85	4	159	7.6
7	5	32	85	5	177	8.5
8	15	32	85	4	166	7.9
9	10	22	70	6	194	9.1
10	0	22	70	5	147	7.4
11	20	22	70	6	194	8.9
12	10	2	70	4	152	7.3
13	10	42	70	6	214	10.2
14	10	22	40	7	204	10.3
15	10	22	100	0	58	3.0

Statistical analysis showed that the variation due to ejaculates and treatments were highly significant, for all the three responses. The most detrimental effect appeared to be due to increasing temperature, which was highly significant for survival and motility. Increasing cell concentrations appeared to have significant beneficial effect on total percent and rate of motile sperm but had little effect on total survival. The interactions among the 3 variables were not significant statistically. From a practical consideration it was deduced that 10-12% yolk, $25\text{-}32 \times 10^6$ total cells per ml. may be the optimums. Although temperature in the vicinity of 60°F may be better, for practical purposes semen can be stored at about 70°F without great loss of motility over the storage periods normally practised in routine A. I. (about a week). Ampoule volumes seem critical for storage of semen in IVT diluent and may alter all results. In two out of the 5 ejaculates, motility persisted beyond two weeks. On the basis of motility observations, some of the semen samples could have been used for A. I. even at the end of two weeks.

(b) Effect of mechanical shaking on sperm in storage

The effect of mechanical shaking of semen samples an inevitable consequence attending all shipments of semen was then tried. Ampoules 1 and 5, 2 and 6, 3 and 7, 4 and 8, 9, 14 and 15 had the same concentra-

TABLE 4

Drop in motility of stored semen subjected to 16-20 hours of mechanical shaking

(average of five ejaculates)

Treatment	% yolk (ml)	Cell Conc. $\times 10^6$/ml.	Initial Motility		Drop in Motility	
			%	Rate	%	Rate
1	5	12	60	3.0	24	1.8
2	15	12	60	3.0	21	1.6
3	5	32	60	3.0	19	1.4
4	15	32	60	3.0	19	1.7
5	5	12	60	3.0	23	1.8
6	15	12	60	3.0	18	1.5
7	5	32	60	3.0	17	1.4
8	15	32	60	3.0	19	1.6
9	10	22	60	3.0	12	1.3
10	0	22	60	3.0	38	2.2
11	20	22	60	3.0	23	1.8
12	10	2	60	3.0	15	1.2
13	10	42	60	3.0	22	1.7
14	10	22	60	3.0	20	1.6
15	10	22	60	3.0	20	1.5

tions of yolk and cells. The ampoules were shaken on a mechanical shaker for 16-20 hours at room temperature (74±6°F). The average decline in percent and rate of motile sperm for 5 ejaculates thus treated are presented in Table 4.

The greatest drop was observed in ampoule No. 10, which had no yolk. Ampoules 1 and 5, 2 and 6, 4 and 8, 3 and 7 (which were duplicates) agreed closely in the motility scores assigned to them. Of ampoules Nos. 9, 14 and 15 (which were triplicates) Nos. 14 and 15 agreed well in their assigned motility scores, but No. 9 showed markedly less drop as also the least drop in percent motility for the entire series. The reason for this discrepancy is not known. It would seem that 10% yolk was adequate in affording about the optimum protection from mechanical shaking. It was felt that the mechanical shaking of the ampoules employed in this experiment was far more rigorous than what would occur under normal conditions of shipment and transport in the field.

Fertility of stored bovine semen preserved with CO_2 gas

For these trials semen 5-6 day old stored in IVT diluent at room temperatures that varied from 60-80°F was used. Semen came from two *Holstein* bulls 'King Jule' and 'Ideal' and the experiment was run between 6th and 18th December 1956 at the Northern Illinois Breeding Cooperative Organization at Hampshire, Illinois. A minimum of 10 first service cows per day with semen from each of the two bulls were insemi-

TABLE 5

Details of Insemination

Technician	Bull	Age of semen in days						Total
		1–2	2–3	3–4	4–5	5–6	6–7	
	Period: December 7 to December 12, 1956							
R.K.	King Jule	5	3	3	5	5	2	23
	Ideal	5	4	4	4	5	3	25
G.A.	King Jule	—	—	2	—	—	—	2
	Ideal	—	—	1	—	—	—	1
	Period: December 14 to December 18, 1956							
R.K.	King Jule	—	3	2	2	—	—	7
	Ideal	—	2	1	2	—	—	5
G.A.	King Jule	5	5	5	4	5	—	24
	Ideal	6	5	5	3	5	—	24
	Total	21	22	23	20	20	5	111

nated over a period of 10-12 days. One technician did most of the inseminations during the first half of the period and the second technician did the major inseminations during the second half period. A short report has already been published (VanDemark and Sharma, 1957). A fuller report is given below. Details of inseminations are given in Table 5.

Out of the 111 cows inseminated, 8 were heifers, 6 were 'repeats' having failed to settle to previous services and some others were suspected for doubtful settling potential. One cow returned to service two days after insemination. Environmental temperatures in the field varied from about 22-80°F and necessary precautions were taken to protect the semen from temperature shock. In Table 6 are given the percent of non-returns during the succeeding four-week periods upto 20-22 weeks.

TABLE 6

Fertility of CO_2 preserved Bovine semen (% of non-returns)

Bulls	Age of semen in days	No. of services	28-39 days (over 4 wks.)	56-67 days (over 8 wks.)	84-94 days (over 12 wks.)	112-126 days (over 16 wks.)	140-154 days (over 20 wks.)
King Jule	1-2	10	90	90	90	90	90
	2-3	11	91	82	82	82	82
	3-4	12	92	75	75	75	75
	4-5	11	72	72	72	72	72
	5-6	10	90	70	70	70	70
	6-7	2	100	100	100	100	100
Total :-	1-7	56	87.5	78.6	78.6	78.6	78.6
Ideal	1-2	11	73	73	73	73	73
	2-3	11	91	64	64	64	64
	3-4	11	82	73	73	73	73
	4-5	9	78	67	67	67	67
	5-6	10	80	70	70	70	70
	6-7	3	100	100	100	100	100
Total:	1-7	55	81.8	70.9	70.9	70.9	70.9
G. Total:	1-7	111	84.7	74.8	74.8	74.8	74.8

The drop between the overall mean percent non-returns for the two bulls from 28-39 days basis to 56-57 days basis was less than 10% (84.7% to 74. 8%). This pattern is normal.

A comparative statement of percent of non-returns (60-90 days) for 111 cows bred with IVT stored semen and 535 cows bred with yolk citrate diluent at 42°F is given in Table 7.

TABLE 7

Fertility of IVT and yolk citrate stored semen

Age of semen in days	Semen in IVT diluent stored at 60–80°F No. cows bred	% NR 60–90 days	Semen in 1 : 3 yolk citrate diluent stored at 42°F. No. of cows bred	% NR 60–90 days
1–2	21	81.0	469	72.0
2–3	22	72.7	43	41.9
3–4	23	73.9	15	40.0
4–5	20	70.0	2	0
5–6	20	70.0	6	0
6–7	5	100.0	—	—
Total :	111	74.8	535	66.9
By bulls :	—		—	—
King Jule	56	78.6	197	73.6
Ideal	55	70.9	338	63.1

Portions of semen samples were shipped simultaneously to the Central Laboratory for tests for duration of motility. All the ejaculates survived for more than two weeks, and the motility was very good. Judging by motility alone, all the samples could have been used for two weeks. Further studies to test the fertility of semen stored for two weeks and more and the investigations leading to the cause of sperm mortality under prolonged storage are desirable.

Summary

1. Possibility of storing semen at room temperatures 60-80°F was investigated using the flow dialysis technique developed by VanDemark and Couturier (1956) and Walton and Dott (1956). Different media were tried.

2. The studies led to the development of a new diluent of bovine semen called 'Illini Variable Temperature' (IVT) dilutor consisting of the following tested ingredients per litre of distilled water :

Sodium citrate dihydrate	20.01	gms.
Sodium bicarbonate	2.01	,,
Potassium Chloride	0.40	,,
Glucose	3.00	,,
Sulfanilamide	3.00	,,
Penicillin	1×10^6	IU.
Streptomycin	1.00	gm.
CO_2 gas	10-15 minutes gassing	

3. Fertility trials carried out on 111 cows using IVT preserved and CO_2 gassed semen samples of 2 Holstein bulls at 60-80°F from 1-7 days of storage gave an overall percentage of 74.8 which compares favourably with the 66.9% obtained in controls comprising 535 cows.

4. Further tests involving larger number of animals and longer periods of preservation are considered desirable.

5. These studies brought out the remarkable role of CO_2 gas in preservation of the life and fertility of bovine spermatozoa at room temperatures (60-80°F).

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NEUROSECRETORY CELLS OF THE NUCLEUS PRAEOPTICUS OF THE BRAIN OF THE FISH, *TILAPIA MOSSAMBICA*

K. K. Nayar and Maya Menon

Department of Zoology, University College, Trivandrum, India

NEUROSECRETORY cells *i.e.*, predominantly glandular neurons, have been shown to exist and to possess important endocrinological functions, in most groups of vertebrates and invertebrates (vide review Scharrer and Scharrer, 1954). In India, work on the morphology and physiology of neurosecretory cells have been attempted chiefly in insects among the invertebrates and to some extent in amphibians and reptiles among the vertebrates (Nayar, 1959). Efforts to obtain information on the cytomorphology of these cells in other animal groups have also been made (Nayar, 1959). However, no work on fishes has so far been attempted in India, and the present paper forms the first contribution towards an understanding of the morphology and histophysiology of the neurosecretory cells of the central nervous system of the fish *Tilapia mossambica*, a species which has become abundant in ponds, streams and paddy fields of Kerala in recent years. The cytomorphology of the well-known region of the praeoptic nucleus of the brain is studied.

Materials and Methods

The present investigation was carried out on fingerlings, 1 to 2 inches in length, obtainable from freshwater tanks of Palghat and Chittur areas of the state. Freshly collected specimens were anaesthetised with ether added on to water in a bowl, and the brains were dissected out and fixed in Bouin's fluid. In some cases the head was cut off from a live fish and fixed entire. In the latter cases the material was decalcified by soaking in 5 to 8% nitric acid in 10% formalin for 4 to 10 days. Sections of 6 μ were cut after processing through methyl benzote containing celloidin. Staining was done in Gomori's chrome alum haematoxylin-phloxine.

Observations

Lying on the sides of the diacoel, nearer the cerebral lobes and above the optic chiasma, are the two praeoptic nuclei of the brain. Each is an extensive tract of conspicuous secretory neurons. The region which ex-

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tends over a distance of 750 μ longitudinally and 500 μ dorsoventrally, shows two distinctive types of cells arranged along a vertical plane. A set of large cells comprise the pars magnocellularis lying in a dorsal plane towards the anterior side. Forming the main bulk of the nucleus praeopticus is the second set of cells which are smaller and occupy a more ventral position ; these constitute the pars parvocellularis.

Pars Parvocellularis :

This region contains a very large number of cells which exhibit the typical neurosecretory pattern of staining. The cells on an average, measure 15 μ in length. The nuclei of the cells are well defined and the cytoplasm shows the presence of stainable colloids coloured blue to black in a variable degree. The axonic processes of these cells are traceable for fairly long distances.

These cells, however, do not show a uniform pattern of histological make up. Differences in the cytomorphology are clearly marked.

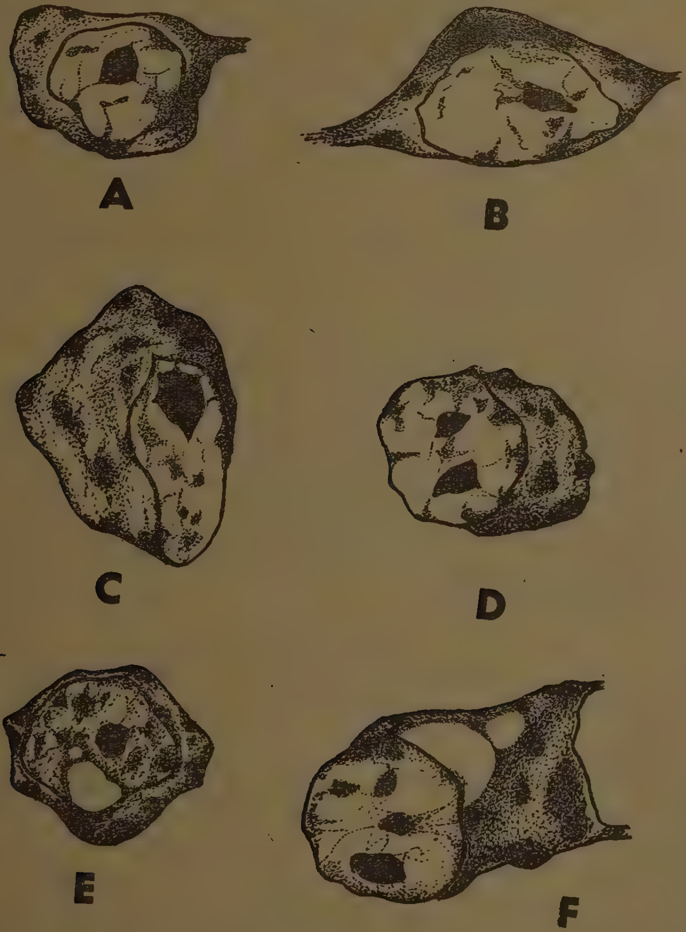
In most of the cells the nuclei appear as perfectly spherical vesicles with faintly marked network of chromonemata. But there is noticeable variation in the size of the nuclei. In several cases, they form large spheres showing up the characteristic pattern (Fig. 1 A). In a few cells, however, having no specific distribution in the praeoptic tract, the nuclei of the cells look enlarged nearly filling the body of the cell (Fig. 1 B). In others, they show a difference in size and shape in that they appear elongated, oval or somewhat compressed (Fig. 1 C).

Typically a nucleus shows a distinct, phloxinophilic nucleolus. But a few cells, especially towards the more ventral part of the praeoptic tract, show two (Fig. 1 D) and sometimes even three nucleoli. All the nucleoli are characteristically phloxinophilic.

Nuclear inclusions form a distinctive feature of the morphology of several of the cells. One (Fig. 1 E) or rarely two to four vacuoles are observable within the nuclei of several cells. But in no case, a colloidal accretion which is distinctly stainable has been observed within the nucleus.

The cytoplasm is generally laden with stainable colloids. They appear coloured blue or bluish black in chrome alum-haematoxylin. The secretory colloids show a clumped pattern, several granules being clumped in groups and distributed evenly in the cytoplasm.

Vacuoles from a very important inclusion of the cytoplasm of these cells (Fig. 1 F). They are of variable size, and show up singly or in groups. They do not have a definite position in relation to nuclei. A few cells show a crescent or halo of a single continuous vacuole around the colloid-filled cytoplasm, showing a region probably originally filled with secreted matter of a different nature.



Camera lucida drawing of different types of cells of the parvacellularis of the brain of *Tilapia mossambica.*

A. normal cell; B. cell with enlarged nucleus; C. cell with compressed nucleus; D. cell with two nucleoi within the nucleus; E. cell with intranuclear vacuole; F. cell with cytoplasmic vacuoles.

Pars Magnocellucelaris :

The cells of this region are larger and show more uniformity in their organisation. They measure on an average 46 μ in length with the axonic part well defined for a third of its length.

It should also be mentioned that several blood capillaries run in the bed where the praeoptic nucleus lies.

Discussion

The teleost neurosecretory system has been studied by some workers (Scharrer and Scharrer, 1954). The variability in the nature of the neurosecretory cells of the diencephalon of fishes has also been recorded in some cases. Most of the observations relate to the intranuclear production of stainable secretory matter and the make up of the cytoplasmic components. In *Tilapia* several differences are observable in the cytomorphology of secretory neurons of the praeoptic region. Enami (1955) has shown in his studies on *Anguilla japonica* that there is a pronounced nuclear secretion, a phenomenon which has been earlier recorded by Scharrer (1934) in the nucleus lateralis tuberis of *Tinca*, by Palay (1943) in the same region of catfishes and by Hild (1949) in the praeoptic nucleus itself of *Tinca*. In *Tilapia*, however, in the fingerling stages studied, such a condition is not seen; the only observable feature is the presence of intranuclear vacuoles. Pillai (1956) has shown in the tadpole stages of *Rana hexadactyla* that the cells of the paraeoptic nucleus shows well defined intranuclear secretory activity.

Summary

1. The praeoptic nucleus of fingerlings of *Tilapia mossambica* consists of a ventral parvocellular and a dorsal magnocellular clusters of neurosecretory cells.

2. The magnocellular region consists of large neurons with a more or less uniform morphological appearance, each cell measuring about 46 μ in length.

3. The parvocellular region consists of a large number of smaller neurosecretory cells, measuring on an average 15 μ length. These neurons show variations in their morphology in exhibiting differences in size and shape of nuclei, number of nucleoi, intranuclear vacuolear vaculoles and cytoplasmic contents. Clumped colloids and vacuoles are observable within the cytoplasm of these cells.

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A STUDY OF CERTAIN BIOCHEMICAL PROPERTIES OF THE PIGEON ADIPOSE TISSUE LIPASE

J. C. George and J. Eapen

Laboratories of Animal Physiology and Histochemistry, Department of Zoology, M. S. University of Baroda, Baroda

THE occurrence of a 'true lipase' in the adipose tissue of the pigeon and certain other vertebrates has been demonstrated employing both histochemical as well as quantitative methods (George and Eapen, 1958 a, b, 1959 a, c, d). The lipase activity in the adipose tissue of the different vertebrates vary considerably. George and Scaria (1959a) studied the properties of the pigeon breast muscle lipase and that of the same enzyme occurring in the pigeon pancreas (1959b). The results of the investigations undertaken to elucidate certain properties of the pigeon adipose tissue lipase are presented in this paper.

Material and Methods

The enzyme material used was the ether-defatted visceral adipose tissue, covering the coils of the intestine, of the pigeon (*Columba livia*). Acetone was found to reduce the activity of the adipose tissue lipase and according to George and Scaria (1959a) the pigeon breast muscle lipase is totally inactivated by acetone. Fiore and Nord (1950) used an ether-defatted dry powder of *Fusarium lini* Bolly as the enzyme material for the study of the lipase of this mould. George and Scaria (1959 a, b) also used an ether defatted powder of the breast muscle and the pancreas of the pigeon for the study of this enzyme. This procedure was found to be highly satisfactory for the present investigation also. The visceral adipose tissue of decapitated pigeons were removed, cut into small pieces and kept in ethyl ether at room temperature for 2 hours with two changes of the solvent. Subsequently this was dried in a vacuum desiccator at room temperature, and stored in cold (4°C). An aqueous extract of this was used as the enzyme solution in all the experiments.

40–50 mg of this defatted tissue was extracted in 4 ml of distilled water in cold (4°C) for 1 hour by grinding it in a mortar. It was then transferred whole to a centrifuge tube and centrifuged for 5 minutes at

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about 2500 r.p.m. and the resulting supernatant used as the enzyme solution. The protein content in 1 ml of this solution was on the average 1.2 mg. Protein was estimated by the micro Kjeldahl steam distillation method for total proteins (Hawk *et al*, 1954). The nitroprusside reaction for sulphydryl groups (-SH groups) and lead-blackening test for sulphur were tried. The enzyme was also treated with excess BAL (British Anti-Lewisite, 2 : 3 dimercaptopropanol) to assess the presence of metals, if any. Metals like iron and copper is known to give a precipitate when treated with BAL (Barron *et al*, 1947a ; Webb and Van Heyningen, 1947).

Lipolytic activity of the extract was determined manometrically in a bicarbonate-carbon dioxide buffer system of pH 7.4 at 37°C (Martin and Peers, 1953). An emulsion of tributyrin (4% v/v) in 0.0148 M $NaHCO_3$, prepared by shaking in a small Erlenmeyer flask with a drop of " Tween 80 " was used as the substrate. Each reaction flask contained 1.5 ml of 0.025 M $NaHCO_3$, 0.5 ml of the substance under test (the various chemicals used) in such concentrations as to give a final concentration as noted below, and 0.5 ml substrate in the side arm in a total fluid volume of 3 ml. The test solutions were introduced into the flasks before the addition of the enzyme, unless otherwise stated. The flasks and manometers were gassed for 3 minutes with a mixture of 95% N_2 and 5% CO_2. After equilibration for 10 minutes in the constant temperature water bath (37°C), the substrate was tipped in and again allowed to equilibrate for another 3 minutes. This period was sufficient to ensure complete mixing of the contents of the flasks. The readings were taken after every 15 minutes for 1 hour. For each experiment a control was run in which 0.5 ml distilled water was added in place of the solution under test. Autohydrolysis was found to be nil. The readings given are after correction for the thermobarometer. The controls produced on the average about 218 μl CO_2, equivalent to the amount of butyric acid liberated.

Results

Effect of various substances

Inorganic salts (Figures 1 and 2) : $CaCl_2$ at all the concentrations tried, activated the enzyme. NaCl, KCl, $MgCl_2$ and NH_4Cl were inhibitory.

Intermediate metabolites (Figure 3) : All the intermediate metabolites tested had a marked inhibitory effect on this enzyme at concentrations 0.0025 M to 0.01 M.

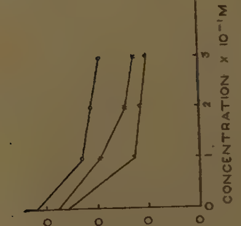

FIG.1

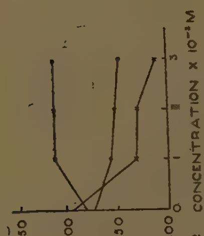

FIG.2

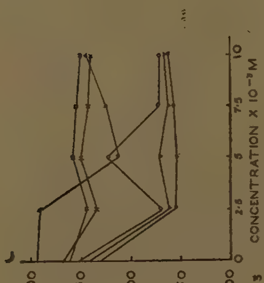

FIG.3

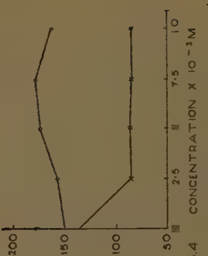

FIG.4

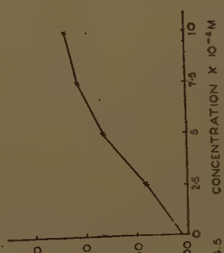

FIG.5

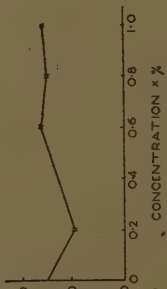

FIG.6

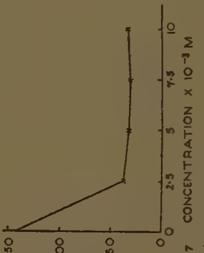

FIG.7

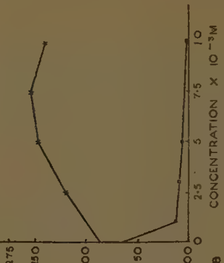

FIG.8

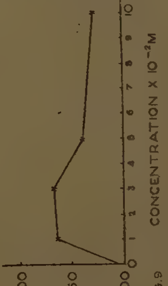

FIG.9

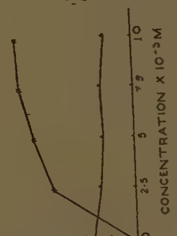

FIG.10

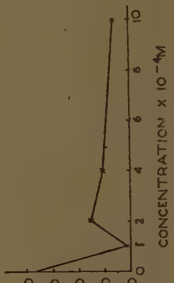

FIG.11

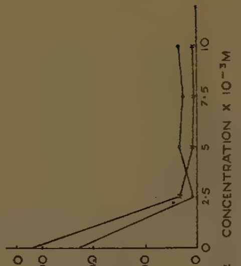

FIG.12

Amino acids : Of the two amino acids tested L-methionine inhibited the enzyme at all the concentrations tried, whereas histidine activated it (Figure 4).

ATP (adenosine triphosphate) was found to activate the enzyme at a progressive rate from 0.00025 M to 0.001 M (Figure 5).

Urethane (Figure 6) : At a concentration of 0.2%, the enzyme was inhibited, while at higher concentrations (0.6%, 0.8% and 1%) there was slight activation.

Sodium taurocholate : (Figure 7) was found to inhibit the enzyme considerably, from 0.0025 M to 0.01 M, the maximum inhibition being about 80% at 0.005 M and 0.0075 M.

Metal chelating agent : 8-hydroxyquinoline is known to bind metals in biological materials (Albert and Gledhill, 1947). The solubility of this substance in water is extremely low. A saturated aqueous solution was prepared (31°C) and 1.5 ml of this solution was added to 1.5 ml of the enzyme solution and incubated at 37°C for 30 minutes. A control was prepared by similarly incubating 1.5 ml of the enzyme with 1.5 ml of distilled water. 1 ml each of these preincubated enzymes was added to each flask. The enzyme was found to be activated about 18% by this chemical.

Thiol reagent : Alkylating agent-Iodoacetate was found to activate the enzyme at concentrations from 0.0025 M to 0.01 M. The maximum activation was about 36% at 0.0075 M (Figure 8).

Explanation of figures :

The effect of the various substances on the pigeon adipose tissue lipase.

Fig. 1 and 2 — Effect of inorganic salts. x, NaCl ; o, KCl ; △, NH_4Cl (Fig. 1). x, $MgCl_2$; □, $CaCl_2$; o. KCl (Fig. 2).

Fig. 3 — Effect of intermediate metabolites. x, succinate ; □, pyruvate ; △, malate ; o, α ketoglutarate ; ▽, lactate ; ●, citrate.

Fig. 4 — Effect of amino acids. x, L-methionine ; o, histidine.

Fig. 5 — Effect of ATP.

Fig. 6 — Effect of urethane.

Fig. 7 — Effect of sodium taurocholate.

Fig. 8 — Effect of iodoacetate and pot. ferricyanide. x, iodoacetate ; □, pot. ferricyanide.

Fig. 9 — Effect of thioglycollate.

Fig. 10 — Effect of glutathione and cysteine. x, glutathione ; △, cysteine.

Fig. 11 — Effect of BAL.

Fig. 12 — Effect of PCMB and $HgCl_2$. x, PCMB ; o, $HgCl_2$.

Oxidizing agent: Potassium ferricyanide was found to be a potent inhibitor. At a concentration of 0.01 M the inhibition was about 39% (Figure 8).

Reducing agents: Monothiol, Thioglycollate (Figure 9) activated the enzyme at all concentrations tested from 0.01 M to 0.1 M.

Glutathione and cysteine: (Figure 10) Glutathione markedly activated the enzyme progressively from 0.0025 M to 0.01 M, while cysteine slightly inhibited it at these same concentrations. At 0.01 M, activation due to glutathione was about 195%.

Dithiol: BAL at all concentrations tested (0.0001 M to 0.001 M) was inhibitory. Maximum inhibition observed was about 63% at 0.0001 M (Figure 11).

Mercaptide forming substances: PCMB (p-chloromercuribenzoate) at all concentrations tested was highly inhibitory. Almost complete inhibition was noted at a concentration of 0.0075 M (Figure 12).

Mercuric chloride: was also an effective inhibitor (Figure 12).

Effect of pre-incubating the enzyme first with $HgCl_2$ and then with BAL. To 1.5 ml of the enzyme solution was added 0.5 ml of 2.4×10^{-4} M $HgCl_2$ and incubated for 15 minutes at 37°C. A control was also similarly incubated side by side, with 0.5 ml distilled water added to 1.5 ml of the enzyme (tube 1). To 1 ml of the former was added 1 ml distilled water (tube 2) and to the remaining 1 ml 6×10^{-4} M BAL (tube 3). To tube 1 was added 2 ml distilled water, so as to make the concentration of the enzyme the same in all the three tubes. All the tubes were again incubated for 15 minutes and 1 ml each of these added to the Warburg flasks and the activity of the enzyme determined as usual. The results are given in table 1. Flask 2 had a final concentration of 10^{-5} M $HgCl_2$ and flask 3 contained 10^{-5} M $HgCl_2$ and 10^{-4} M BAL.

TABLE 1

The effect of pre-incubating the pigeon adipose tissue lipase with $HgCl_2$ and BAL

Activity, μl CO_2 produced		
Control, without addition (1)	Enzyme+ $HgCl_2$ 10^{-5} M (2)	Enzyme+$HgCl_2$ 10^{-5} M +BAL 10^{-4} M (3)
259.9	196.4	120.9

$HgCl_2$ was found to inhibit the enzyme about 24%. A further inhibition of about 29% was brought about by the addition of BAL. *The effect of pre-incubating the enzyme first with BAL and then with $HgCl_2$.* 1.5 ml of the enzyme solution was incubated for 15 minutes with 0.5 ml of 6×10^{-4} M BAL at 37°C. A similarly incubated preparation containing 1.5 ml enzyme and 0.5 ml distilled water was used as control (tube 1). To 1 ml of the former was added 1 ml distilled water (tube 2) and to the remaining 1 ml, 1 ml of 2.4×10^{-4} M $HgCl_2$ (tube 3). 2 ml of distilled water was added to tube 1 and all the tubes incubated for a further period of 15 minutes. 1 ml each of these was added to separate flasks and the activity noted. The final concentration of BAL and $HgCl_2$ was 2.5×10^{-5} M and 4×10^{-5} M respectively (table 2).

TABLE 2

Effect of pre-incubating the pigeon adipose tissue lipase with BAL and $HgCl_2$

Activity, µl CO_2 produced		
Control, without addition (1)	Enzyme + BAL 2.5×10^{-5} M (2)	Enzyme + BAL 2.5×10^{-5} M + $HgCl_2$ 4×10^{-5} M (3)
271.2	179.3	137.6

The enzyme was inhibited about 34% by BAL and a further inhibition of 15% was noted due to the addition of $HgCl_2$.

Effect of pre-incubating the enzyme first with PCMB and then with glutathione. 1.5 ml of the extract was incubated with 0.5 ml of 6×10^{-3} M PCMB for 15 minutes at 37°C. To another 1.5 ml portion of the extract was added 0.5 ml distilled water (tube 1) and incubated as above. This served as control. To 1 ml of the former was added 1 ml distilled water (tube 2) and to the remaining 1 ml, 1 ml 1.44×10^{-3} M glutathione (tube 3). 2 ml of distilled water was added to tube 1 and all the three tubes incubated for a further period of 15 minutes. 1 ml each of these preparations was added to separate flasks and the activity noted as usual. The final concentration of PCMB and glutathione in the flasks were, 2.5×10^{-4} M and 2.4×10^{-4} M respectively (table 3).

TABLE 3

Effect of pre-incubating the pigeon adipose tissue lipase with PCMB and glutathione

Activity, µl CO_2 produced		
Control, without addition (1)	Enzyme + PCMB 2.5×10^{-4} M (2)	Enzyme + PCMB 2.5×10^{-4} M +glutathione 2.4×10^{-4} M (3)
163.9	35.3	192.2

The enzyme was inhibited about 79% by PCMB. The inhibition was completely reversed by glutathione and an activation of about 17% was also brought about.

The nitroprusside reaction for -SH groups and lead-blackening test for sulphur gave negative results. The enzyme solution when treated with excess BAL, did not yield any precipitate thereby indicating the absence at least in easily detectable quantities of metals like iron and copper.

Discussion

The extensive studies on lipases and esterases by the schools of Willstater and Rona are well known. The literature upto 1950 has been reviewed by Ammon and Jaarma (1950). It is generally agreed, that the hydrolysis of tributyrin is effected by 'true lipase' (Martin and Peers, 1953). In the manometric system, the pancreatic lipase hydrolyses tributyrin readily, but olive oil is not acted upon (George and Scaria, 1959b). Martin and Peers (1953) made a similar observation with regard to oat lipase. The latter authors explained the non-hydrolysis of olive oil in a manometric system as due to the predominance of water in the system. Moreover lipases from various sources differ from each other with regard to their solubility, specificity and kinetic properties (Martin and Peers, 1953). From the study of the properties of the pigeon breast muscle lipase (George and Scaria, 1959a) and pancreatic lipase (George and Scaria. 1959b), it appears that every enzyme may be adapted for maximal activity in the respective physiological environment in which it occurs. Our present study also tends to support such a contention.

Sodium taurocholate considerably inhibited the enzyme. All the Krebs cycle intermediates and lactate, also acted as inhibitors. The pigeon pancreatic lipase has been reported to be activated by sodium taurocholate while the breast muscle lipase of the same animal, inhibited by it (George and Scaria, 1959a, b). The Krebs cycle intermediates and lactate activated the breast muscle lipase of the pigeon while the pancreatic lipase was inhibited (George and Scaria, 1959a, b). The activity of the adipose tissue lipase is intracellular and its physiological environment should contain normally, only small quantities of bile salts or intermediate metabolites. Bile salts occur in appreciable quantities in the physiological environment of the pancreatic lipase while the intermediate metabolites in that of the breast muscle lipase. ATP was however, found to activate the adipose tissue lipase. As far as we are aware, we have no information available on the levels of the various phosphate compounds occurring in the adipose tissue of the pigeon. But it is noteworthy that considerable quantities of ATPase was found to be present in this tissue (George and Eapen, 1959b). No cation requirement if any, could be demonstrated by adding these substances. Added cations had only an inhibitory effect, except Ca, which showed an activation.

The negative results obtained with nitroprusside reaction for sulphydryl groups and the lead-blackening test for sulphur tend to show that the enzyme does not contain either-SH groups or disulphide groups (–S–S– groups) in its protein. A similar observation was made with regard to the pigeon breast muscle lipase (George and Scaria, 1959a) and the pancreatic lipase (George and Scaria, 1959b). It should however, be pointed out that there are different types of –SH groups occurring in the native proteins (Barron, 1951). Of these, the "freely reacting-SH groups" readily react with nitroprusside and ferricyanide. The second variety, "sluggish-SH groups" react with more powerful reagents such as PCMB. In certain instance ferricyanide is also known to react with the latter type. The third type. "masked-SH groups", are in general not connected with enzyme activity, since they are so protected as to require protein denaturation to become reactive. But the enzyme activity is destroyed by protein denaturation (Barron, 1951). Potassium ferricyanide, an oxidizing agent, inhibited the adipose tissue lipase. Enzyme inhibition by ferricyanide is assumed to be due to the oxidation of –SH groups to the disulphide (Barron, 1951). Iodoacetate, an alkylating agent, activated the enzyme. It is known that some thiol enzymes (shown to possess –SH groups necessary for enzyme activity with the aid of oxidizing and mercaptide-forming agents) are not inhibited at all by iodoacetate (Barron, 1951).

Barron (1951) has classified pancreatic lipase and esterases under sulphydryl enzymes. Ions of heavy metals such as Hg are said to inactivate such enzymes by combining with reactive –SH groups, forming mercaptide compounds. Such inactivation can be reversed by the addition of BAL because of its greater affinity for the metal. It must be noted, however, that BAL because of its powerful reducing power, inhibits enzyme reactions which are independent of –SH groups in the protein (Barron, 1951). Both $HgCl_2$ and BAL considerably inhibited the adipose tissue lipase. Due to the oxidation of dithiols, –SH enzymes are known to be inhibited (Barron *et al*, 1947b). As is evident from the results (table 1) BAL was not found to reverse the inhibition brought about by $HgCl_2$. Furthermore, due to the cumulative effect of these two substances, enhanced inhibition was the result, when both were present simultaneously (table 1 and 2). Inhibition of the adipose tissue lipase by $HgCl_2$ and PCMB, appears to be due to their reactivity towards –SH groups. The reversal of PCMB inhibition by glutathione supports this view. It has been reported that whenever thiol groups are attacked by agents which destroy the –SH groups, glutathione will restore the –SH groups by withdrawing the heavy metal or by reducing the oxidized –SH groups (Barron, 1951). According to George and Scaria (1959a, b), the inhibition of the pigeon breast muscle and pancreatic lipase is not due to reaction with –SH groups, but is due to the binding of reactive NH_2 groups of the protein. This latter possibility may be ruled out in the present instance due to the fact that PCMB inhibition is totally reversed by glutathione. PCMB is said to be the most powerful inhibiting agent for –SH groups and the most specific when accompanied by reactivation (Barron, 1951). Based on Singer's observations Barron (1951) states, " whereas inhibition of D-amino acid oxidase by PCMB is dependent on the substrate used, the inhibition of lipase depends on the molecular size of the substrate. The larger the substrate molecule, greater the inhibition produced by thiol reagents. " He believes that in such cases as in lipase, " the –SH groups do not contribute to the activity of the enzyme and that the inhibition is due to the steric interference with the approach of the larger substrate molecules to the active surface of the lipase ". This explanation appears doubtful.

Of the four reducing agents tested, thioglycollate and glutathione activated the enzyme, while cysteine and BAL considerably inhibited it, especially the latter. Thiol groups of proteins which have been converted into disulphides can be easily reduced on addition of a large excess of soluble thiol compounds (thio acids, cysteine, glutathione, BAL and other dithiols), and the effectiveness of these vary greatly (Barron, 1951). That the activation of the enzyme by thioglycollate and glutathione is due to this, is doubtful. Another possibility is, that activation may be due to the binding of inhibitory metal ions present in the enzyme solution. The pigeon pancreatic lipase is shown to be activated by glutathione (George and Scaria, 1959b) while cysteine activated the breast muscle lipase of the same animal (George and Scaria, 1959a). The ability of the above mentioned substances to combine with different metals vary considerably (Barron *et al*, 1947b ; Webb and van Heyningen, 1947). That the inhibition is not due to the combination with metals of the enzyme protein, forming mercaptides, is borne out by other results. First among these is the absence of any precipitate on treatment of this enzyme with BAL. BAL in combination with a number of metals, like Fe, Pb, Bi, Cu, Co, Ni, is known to give coloured insoluble complexes (Barron *et al*, 1947a; Webb and van Heyningen, 1947). Second is that, 8-hydroxyquinoline which is a metal-chelating agent (Albert and Gledhill, 1947) did not inhibit the enzyme, but curiously enough activated it. This indicates the lack of a metal in the enzyme protein. As has been mentioned earlier, activation by this chemical also may be due to the binding of some inhibitory metal ions. This, however, is not conclusive.

In conclusion the following may be stated :

1. From the data available from the present experiments it is evident that an aqueous extract of ether-defatted adipose tissue is active by itself and is not dependent on any added substances.
2. The adipose tissue lipase does not contain a metal in its enzyme protein and it does not require any metal ions for activity.
3. This enzyme appears to be an -SH enzyme or one requiring -SH or -S-S- for activity.

Summary

Certain biochemical properties of lipase in aqueous extracts of ether-defatted dry adipose tissue of the pigeon was studied in a manometric system, using tributyrin as substrate. No cation requirement for the enzyme activity could be demonstrated. This enzyme was inhibited by sodium taurocholate, Krebs cycle intermediates and lactate. PCMB and $HgCl_2$ were both inhibitory. 8-hydroxyquinoline activated the enzyme, so also ATP. Glutathione activated the enzyme while cysteine was found

to be inhibitory. The inhibition by PCMB was reversed completely by the addition of glutathione. BAL was also inhibitory. The enzyme appears to be an –SH enzyme or one requiring –SH or –S–S– for activity. It is found that the enzyme activity is not dependent upon the presence of metal or metals.

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ON THE COMPOSITION OF THE SCALES AND SKIN OF *BOLEOPHTHALMUS BODDAERTI* PALL AND *MUGIL DUSSUMIERI* (VAL)

(MISS) P. AMBUJA BAI AND (MISS) M. KALYANI

Marine Biological Station, Porto-Novo, S. India

DURING recent years it is increasingly realised that there is need for detailed investigations on the chemical composition of fish tissues. This is partly because the investigations in the past were almost exclusively confined to fish muscle. The investigations in the past also failed to take note of the fact that the concentration of the chemical constituents of fish tissues is likely to vary considerably from one type of fish to another, depending on several factors.

The skin is known to play a very significant role in the physiology of the fish. But comparatively little information is available regarding the chemical composition and the little that is known indicates that it is no less interesting than the muscle. This is evident from the comparative values for the constituents of the flesh and skin as given by Van Oosten (1957) for cod. On a dry weight basis these are as follows:

	Protein	Ash	Fat
Flesh	87%	5.1%	3.4%
Skin	88.5%	8.2%	2.3%

It is known that considerable variation occurs in the chemical composition of the muscles of fish even among the individual fish of the same species. This must hold true for skin also, as remarked by Van Oosten (1957), but we know nothing of the variability, not to speak of the factors governing it.

The same remarks will apply to the composition of fish scales. Vinogradov (1953) in his classic work has recorded the mineral composition of the scales of six species of fish. Takashi and Shikowa (1953) studied the nitrogenous and mineral constituents in one species, and Obata, Igarashi and Ishida the guanine content in one species. The organic constituents of the scales of eight species of fish have been studied by Green and Tower (1902). Ney, Deas, and Tarr (1950) have determined qualitatively some of the essential amino acids of the scales of *Clupea*

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pallasii and more recently Solomons (1955) has investigated the proteins with special reference to N-terminal amino acids of ichthylepidin in the scales of the pilchard. But practically in all these studies the variability in the concentration of the chemical constituents with the individual fish and in relation to ecological factors and physiological state of the fish has been completely overlooked, and no mention is made even of the number of specimens which were analysed.

The present account dealing with the total mineral content, calcium content and the amino acids of the scales of two species of fish, *Boleophthalmus boddaerti* and *Mugil dussumieri* is an exploratory step in understanding the variation of some of the chief constituents in connection with a project under investigation regarding the comparative physiology and composition of the skin and scales of representative Indian fishes. One of the authors (Kalyani) studied the skin and scales of *Boleophthalmus* and the other author (Ambuja Bai) the skin and scales of *Mugil dussumieri*.

Material and Methods

Specimens of the two species of fish investigated were obtained from the Vellar estuary. The determination of ash and calcium in the scales was made in each of seven specimens in a sample of males of each species. The samples of the two species were more or less alike. The determination of ash and calcium in the skin was made in four specimens of each species. The nitrogen estimation was done only in few specimens, three of *Boleophthalmus* and two of *Mugil*.

For the determination of ash and calcium content, the scales and skin were removed separately from the body, rinsed in water, and any adhering tissues were carefully removed. The scales were also washed in dilute KOH, then washed again with distilled water. A few milligrams of the scales and skin were separately dried to constant weight at 100°C and the former ashed in a muffle furnace at 600°C, and the latter at 200°C.

For determining the calcium content, the method given by Hawk *et al* (1954) was adopted. For the determination of nitrogen in the skin and scales the microkjeldahl (Hawk *et al*) method was adopted. Protein in skin and scales was calculated from the nitrogen values which were multiplied by 6.025, instead of the conventional 6.25, as the former factor is regarded as more satisfactory as Love (1957) has discussed.

For the study of the amino acid composition, the material was hydrolysed with 6N HCL by autoclaving the samples with an excess of dilute

HCL for about 8 hours, at a pressure of 30 lbs. The acid hydrolysates were prepared for chromatographic analysis in the usual manner. Uni-dimensional and also two dimensional chromatographic analysis was carried on Whatman filter paper No. 1. For unidimensional chromatograms N-Butanol, acetic acid and water mixed in the ratio 24 : 6 : 10 was used, and for two dimensional chromatograms buffered phenol was used as the second solvent. 0.25% ninhydrin was used as the colour developing agent.

Observations and discussion

The results of the analyses of scales and skin of the two species of fish are shown in Tables 1 to 3.

TABLE 1

Ash percentage value of scales and calcium percentage in ash of scales. Ash values per 100 gms. dry weight of scales and calcium as % of ash.

	Boleophthalmus boddaerti			*Mugil dussumieri*		
Specimen No.	Ash	Organic Matter	Calcium	Ash	Organic Matter	Calcium
1	35.550	64.450	34.640	41.640	58.400	35.400
2	44.000	56.000	37.980	34.600	65.400	42.850
3	36.560	63.440	33.063	58.650	41.350	35.090
4	41.390	58.610	37.370	39.010	60.990	39.900
5	48.000	52.000	24.130	44.700	55.300	36.800
6	40.000	60.000	22.434	55.770	44.230	33.640
7	47.640	52.360	34.440	54.110	45.990	47.310
Total	293.140	406.860	224.057	328.440	376.660	270.990
Mean	41.877	58.123	32.008	46.92	53.094	38.710
Standard Deviation	4.95	9.93	6.25	10.10	9.50	4.60
Standard Error of S.D.	1.3	1.3	1.7	2.6	2.9	1.2
Coefficient of Variation	11.8	8.5	19.5	21.5	17.9	11.9

't' for differences in the Mean values for ash 2.5 ; P = 0.025

,, ,, ,, ,, ,, ,, ,, Calcium 2.78 ; P = 0.018

TABLE 2

Ash content per 100 grams of dry weight of skin and % of calcium in ash of skin

	Boleophthalmus bodderti			*Mugil dussumieri*		
Specimen No.	Ash	Organic Matter	Calcium % of ash	Ash	Organic Matter	Calcium % of ash
1	3.33	96.67	98.66	3.11	96.89	70.10
2	6.41	93.59	66.60	3.66	96.34	94.300
3	6.41	93.59	66.60	4.30	95.70	43.53
4	5.80	94.20	44.20	7.08	92.92	43.53
Total	21.95	378.05	276.06	18.15	381.90	251.46
Mean	5.487	94.512	69.045	4.337	95.475	62.866
Standard Deviatin	1.450	1.4	22.500	1.760	1.7	24.5
Standard Error of S.D.	0.51	—	9.0	0.6	—	8.7
Coefficient of Variation	26.40	1.50	32.5	38.8	1.86	37.7

TABLE 3

Percentage of total nitrogen and protein in the scales and skin of Boleophthalmus boddaerti and Mugil dussumieri

	SCALES				SKIN			
	Boleophthalmus		*Mugil*		*Boleophthalmus*		*Mugil*	
	N	P	N	P	N	P	N	P
1	9.240	55.671	9.564	57.524	14.280	85.990	14.700	88.570
2	9.240	55.671	9.564	57.524	14.280	85.990	14.700	88.570
3	8.400	50.610	8.700	54.417	—	—	—	—
Mean	8.993	53.959	9.276	56.488	14.280	85.990	14.700	88.570

(a) *Ash and Calcium.*

It will be seen from Table 1 that the scales in the two species have different mean values for ash and calcium. The mean value for ash content in the scales of *Mugil* is 46.92 gms per 100 gms of dry weight of scales, and in the scales of *Boleophthalmus* 41.87. Similarly the mean percentage of calcium in the ash of the scales is higher in *Mugil*, being 38.71 in *Mugil*, and 32.00 in *Boleophthalmus*.

With regard to the skin also the mean values for ash and calcium differ in the two species. But in this case, it is *Boleophthalmus* that has higher mean values for ash as well as calcium.

Comparing the concentration of the constituents in the scales and skin in each species, we find that the skin has a much lower percentage of ash than the scales, but the percentage of calcium is higher in the ash of the skin than in the ash of the scales in both species. This indicates that in the skin the major part of the mineral content is calcium, but the scales contain besides calcium an appreciable quantity of other mineral matter.

Another feature which is evident from the data is that the variation of ash and calcium in the scales is different in the two species. The standard deviations and coefficients of variation of the two samples are different in the two species. The calcium concentration in the ash of scales shows greater variation in *Boleophthalmus*, but the ash content has greater variation in *Mugil*. It may also be observed that there is similarity between the variation of calcium in one species and the ash in the other, and vice versa. This would mean that the scales of the two species differ in the concentration of organic constituents also. It must be admitted, however, that the parameters for variation in the two samples have high values, which might mean that the samples investigated are not adequate and sufficiently unified for arriving at final conclusions.

With regard to the skin, it is in *Mugil* that relatively greater variation is seen in ash as well as calcium content. But here again the inadequacy of the sample must be borne in mind.

Finally we have to consider whether the differences in the corresponding mean values are significant. The 't' values for the differences between the means for ash and calcium in the scales of the two species indicate significance. But the differences in regard to the mineral constituents of the skin are not significant. On the other hand the differences between the corresponding mean values for the scale and skin constituents in both species are significant.

(b) *Nitrogen*

The total nitrogen values are shown in Table 3. The nitrogen (and protein) values differ only slightly in the two species. But the skin has a higher nitrogen content than the scales.

(c) *Amino Acids*

The proteins of the scales of the two specimens of fish, chiefly with reference to ichthylepidin and collagen have been under investigation. In the present note, hewever, only the amino acid composition of the acid hydrolysates of the whole scales and skin is given. We have not been able to detect so far any qualitative differences in the amino acid composition between the scales of the two species. This holds true for the skin also. The amino acids thot have been detected so far are (1) Leucines. (2) Phenylalanine. (3) Methionine. (4) Tyrosine. (5) Proline. (6) Alanine. (7) Glutamic acid. (8) Threonine. (9) Hydroxyproline. (10) Glycine. (11) Serine. (12) Aspartic acid. (13) Arginine. (14) Asparagine. (15) Histidine. (16) Lysine. Cystine is absent in both scales and skin.

Conclusion

The present study indicates that the ash and the calcium content of scales and skin of the fish investigated is very variable, and there is the probability of each species having its characteristic mean and range of variation in respect of some of chief chemical constituents of scale and skin. The variation in the composition of these structures in relation to physiological and ecological conditions is worth precise investigation, and this is in progress.

Regarding the amino acids, Ney, Deas and Tarr (1950) have mentioned only the essential amino acids from the standpoint of nutritive value. Solomons (1955) has given the amino acid content of ichthylepidin and gelatin of the pilchard. In the present study it is found that phenylalanine and arginine occur in addition to the amino acids mentioned for pilchard.

Acknowledgements

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Summary

1. The ash content, percentage of calcium in the ash, total nitrogen and the amino acids of the scales and skin of *Boleophthalmus boddaerti* Pall. and *Mugil dussumieri* (Val.) have been investigated.
2. The skin has a higher total nitrogen content and a lower ash content than the scales.
3. The percentage of calcium in the ash is higher in the skin than in the scales.
4. The two species indicate significance in the differences in their calcium and ash content of the scales. The variation of these constituents is different in the two species.
5. There are no significant differences between the two species with regard to the variation in the ash and calcium content in the skin.
6. But between the skin and scales in each species there are significant differences in the variation of calcium and ash content.
7. The two species do not differ in the amino acids of the scales and skin.

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A HISTOCHEMICAL STUDY OF THE ATRIOVENTRICULAR BUNDLE (BUNDLE OF HIS) AND THE MYOCARDIUM OF THE SHEEP HEART

J. C. George and P. Thomas Iype

Laboratories of Animal Physiology and Histochemistry, Department of Zoology, M. S. University, Baroda, India

(1 Plate)

THE atrioventricular bundle differs from the cardiac muscle, histologically as well as physiologically. The Purkinje fibres which are regarded as the structural units of the bundle of His are larger than the cardiac fibres and are often characterized by the presence of double nuclei in them. Functionally the bundle differs from the cardiac muscle in being conductile. It is also known to be rich in glycogen (Truex and Copenhaver, 1947 ; Field, 1951 ; Poppen *et al*, 1953), potassium (Poppen *et al*, 1953) and cholinesterase (Carbonell, 1956). In addition to its role in conduction and of storage of glycogen in the heart, a possible new function, that of synthesis of fat, has been recently suggested (Kuwabara and Cogan, 1959). These authors have shown experimentally that the Purkinje fibres appear to possess the ability to synthesize sudanophilic fat, which is not shared by the cardiac muscle, thereby suggesting the existence of a distinct fat metabolism in this tissue. In the light of these findings, we were encouraged to undertake the present study of the bundle of His and the cardiac muscle with a view to investigate the problem of lipogenesis by the Purkinje fibres by exploring histochemically the actual localization of some of the enzymes, particularly lipase.

The sheep heart was chosen as the material because in this the fibres of the atrioventricular bundle are bigger (30µ) than those of the laboratory animals like rat (9µ) and rabbit (10µ) (Davies and Francis, 1952), and also of the advantage in the bundle being enclosed in a connective tissue sheath which makes it easily discernible.

Material and Methods

Fresh sheep hearts from the slaughter house, each excised immediately after killing the animal and placed in a polyethelene bag covered

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with ice were used within ten minutes during which time the material was transported from the slaughter house to the laboratory. The heart was dissected immediately and a block of the interventricular septum nearer to the auricles was cut along with a portion of the auriculo-ventricular bundle. This block was fixed on the stage of a carbondioxide freezing microtome in such a way as to cut the bundle transversely, and frozen immediately. Sections were cut for the different histochemical observations on enzyme activity and blocks of the tissue were also cut from the same and fixed in appropriate fixatives for fat, glycogen, mitochondria and general histology. In order to see if there were any distinct changes as a result of the time lag in transport, tissues were also fixed in the different fixatives at the slaughter house itself, immediately after removal of the heart from the animal. All the experiments were repeated on different hearts.

Lipase :

The 'Tween' method of Gomori (1953) for lipase using "Tween 80" (Atlas Powder Co) as substrate was followed with certain modifications. Frozen sections of 40μ – 80μ thickness were cut high in the interventricular septum and dropped directly into 6% formalin neutralized with Na_2HPO_4 and maintained at 4°C for fixation. After fixation for 3–4 hours the sections were washed in a gentle stream of running water for an hour and mounted on clean albumenized glass slides and dried. Control sections were boiled for ten minutes in water to inactivate the enzyme. All the sections were then coated with a thin layer of 10% aqueous gelatin. The rest of the procedure was the same as described by George and Iype (1960).

Succinic Dehydrogenase :

Histochemical localization of succinic dehydrogenase was demonstrated using neotetrazolium as the hydrogen acceptor. George and Talesara (1960) have reviewed the earlier literature and also pointed out that the activity of this enzyme in tissues containing very little succinic dehydrogenase activity hardly demonstrable by histochemical methods under aerobic conditions, could be successfully demonstrated when carried out under anaerobic conditions. The present experiments were conducted under both the conditions.

Fresh frozen sections of 40μ thickness were cut from the same tissue block and dropped in ice-cold phosphate buffer for nearly ten minutes in order to remove the endogenous substrate from dehydrogenase activity.

Meanwhile nitrogen was bubbled for 5 minutes through the incubation medium (George and Talesara, 1960) which consisted of 2.5 ml PO_4 buffer (0.1M, pH 7.6), 0.6 ml sodium succinate (0.5M, neutralized to pH 7.6), 0.5 ml $CaCl_2$ (0.004M), 0.5 ml $AlCl_3$ (0.004M), 0.3 ml $NaHCO_3$ (0.6M, freshly prepared), 0.05 ml $MgSO_4$ (0.005M), 0.5 ml NaCN (0.03M, neutralized to pH 7.6), 1 ml neotetrazolium (3 mg/ml, freshly prepared) and 0.05 ml methylene blue (2 mg/ml). The sections were placed into the incubation mixture and incubated for 15-30 minutes. The sections were then washed well in phosphate buffer, fixed in 10% neutral formalin in cold for 4-6 hours and mounted in glycerine jelly, after washing away the formalin. The preparation was immediately examined and microphotographed. Control sections were incubated in an incubation medium without the substrate.

Cytochrome oxidase :

G-Nadi reaction for cytochrome oxidase (Pearse, 1954) was tried on 40μ thick sections from the same tissue block.

Fat :

Thin tissue blocks were fixed in Baker's calcium formol for 24 hours, postchromated for 18 hours at room temperature and 24 hours at 60°C in potassium dichromate solution (Baker, 1946). After washing they were embedded in gelatin and sections of 15μ thickness were cut and mounted. The sections were stained with saturated Sudan Black B solution in 70% alcohol. Some of the sections were stained with Oil Red O in 1% Tween 40 dissolved in 30% alcohol (Bell, 1959).

Glycogen :

Glycogen was stained by the PAS technique.

Mitochondria :

4μ thick paraffin sections of the tissue fixed in Altman's fluid were stained with Anilin Fuschin (20% Acid Fuschin in saturated solution of Anilin oil), 0.5% Toluidine blue and differentiated in 0.5% Aurentia in 70% alcohol (Gray, 1954). They were rapidly dehydrated, cleared in xylol and mounted in Gum dammar. Adequate care was taken to avoid the settling of bacteria on the slides, because of their affinity for the stain.

For general histology paraffin sections fixed in Bouin's fluid were stained with Heidenhain Azan Stain (Gurr, 1956).

Results

Lipase :

Lipase was found to be distributed more or less equally both in the bundle of His and in the cardiac muscle (Fig. 1). The possibility of diffusion of the black precipitate from the original sites of lipase activity is remote because of the gelatin coating (George and Iype, 1960). This is further supported by the fact that no precipitate was seen in the region of the connective tissue sheath covering the bundle, since if there was any diffusion from the cardiac muscle to the bundle of His, there should have been at least some precipitate on the connective tissue sheath also. Unlike the cholinesterase which has been shown to be localized only in the periphery in the lamb conductive system (Carbonell, 1956) the lipase was seen all over the Purkinje fibres.

Succinic Dehydrogenase :

The concentration of succinic dehydrogenase was considerably less in the bundle than in the cardiac muscle. (Fig. 2 and 3). The cardiac muscle showed a large number of deep purple diformazan granules whereas in the bundle the number of granules was comparatively small and the intensity of the colour was also much less. Rutenburg *et al* (1953) studied the succinic dehydrogenase activity in different tissues of six mammals and reported highest activity in the heart. They also showed that the heart like other tissues was stained more intensively under anaerobic conditions.

Cytochrome Oxidase :

Cytochrome oxidase also showed the same pattern of distribution as that of succinic dehydrogenase in being more in the cardiac muscle and less in the bundle of His.

Fat :

Fat was found to be less in the bundle when stained with Sudan Black B and also with Oil Red O in Tween 40 alcohol solution (Fig. 7 and 8). Fat in the bundle was invariably seen towards the periphery of the Purkinje fibres.

Mitochondria :

Mitochondria were comparatively much less in the bundle than in the cardiac muscle (Fig. 4, 5 and 6). In the Purkinje fibres the mitochondria were seen chiefly towards the periphery (Fig. 4 and 5). This is in agreement with the findings of Muir (1957).

Nuclei :

In sections stained with Azan stain, the double nuclei of the Purkinje fibres were clearly seen and the connective tissue was also stained.

Discussion

The results obtained from the present investigation indicate that the metabolism of the conducting system of the heart and the myocardium are not identical. The bundle contains a very high amount of glycogen and very little fat, suggesting a predominently carbohydrate metabolism distinct from that of the rest of the heart. The cardiac muscle has a high fat content. The high lipase activity in the cardiac muscle is also suggestive of a high level of fat metabolism in this muscle. The role of lipase in bringing about the degradation of fat into fatty acids and glycerol in the cardiac muscle is of special significance in the light of Bing's findings (1956) that fatty acids form the chief energy fuel for the contraction of the heart. Moreover our recent observation (1959) that there is a direct relationship between the heart rate and the lipase activity in the chick heart during development also points to the important role of lipase in the metabolism of the cardiac muscle.

Microphotographs of sections of the sheep heart through the bundle of His and the adjacent myocardium.

FIG. 1 Section treated for the demonstration of lipase activity in the bundle of His (seen in the centre surrounded by a clear area of connective tissue) and the cardiac muscle surrounding the bundle. Note the equally high concentration of the precipitate in both the above areas and also a few fat globules at the periphery of the bundle.

FIG. 2 Section treated for the demonstration of succinic dehydrogenase activity. Note the low concentration of the enzyme in the bundle as opposed to the myocardium.

FIG. 3 Higher magnification of the section in fig. 2 showing granules of diformazan and a couple of fat globules at the periphery of the bundle.

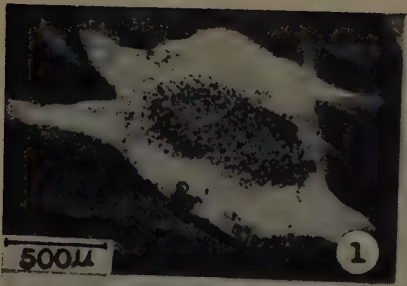
FIG. 4 Section treated for the exposition of the mitochondria. The area within the circle shows a portion of the bundle of His and that in the square a portion of the myocardium.

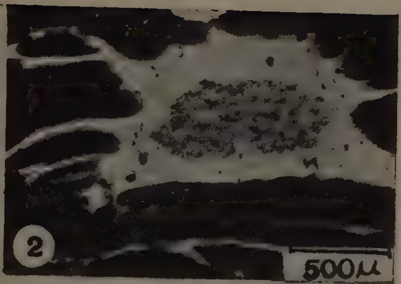
FIG. 5 The region denoted by circle in fig. 4 is presented under high magnification (oil immersion). Notice the distribution of the mitochondia towards the periphery of the Purkinje fibre.

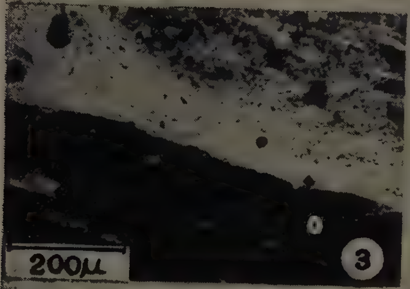
FIG. 6 The region denoted by the square in fig. 4 is presented under high magnification (oil immersion). Note the greater concentration of mitochondria here in comparison with the region in fig. 5

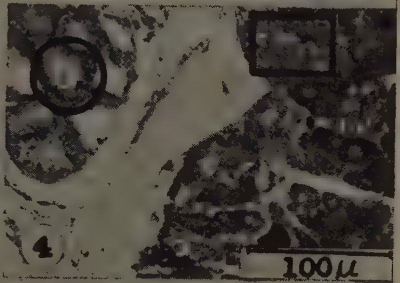
FIG. 7 Section treated with Sudan Black B for fat. Note the high concentration of fat in the myocardium.

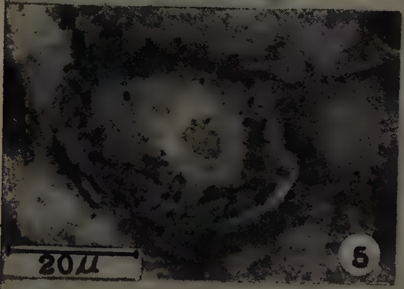
FIG. 8 The same section in fig. 7 is magnified.

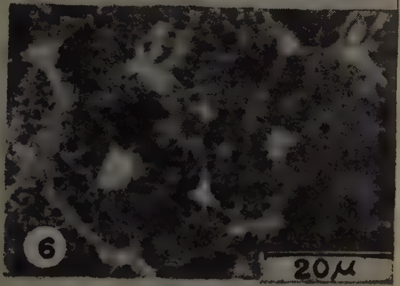


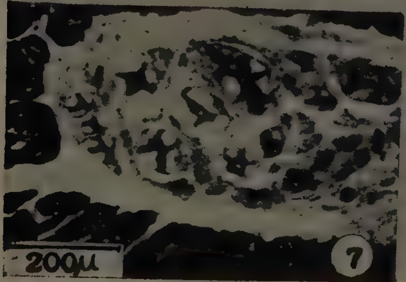












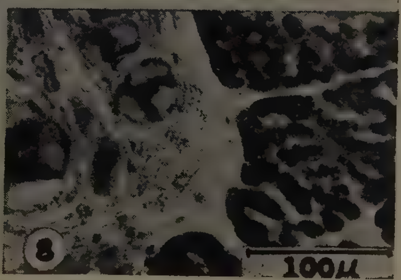


Plate 1

J. C. GEORGE and P. THOMAS IYPE

The occurrence of an equally high concentration of lipase in the bundle of His where the amount of fat present is considerably less, however, needs to be explained. The presence of a certain enzyme in a tissue may be considered as an indication of the occurrence of its specific substrate to be metabolized there. It is well-known that lipase can synthesize fat and also split up fat into its component fatty acids and glycerol. If the latter role of lipase is predominent in the bundle of His, it is logical to expect a high level of activity of the oxidative enzymes there so that the products of lipolysis could be oxidized to yield energy. But it has been shown that the level of the oxidative enzyme activity in the bundle of His is considerably low. On the other hand lipase activity as well as activity of the oxidative enzymes is comparatively very high in the myocardium. These observations lead us to the conclusion that the occurrence of lipase in the bundle of His is chiefly for the synthesis of fat. This is in accordance with the finding of Cogan and Kuwabara (1957) that in the rabbit heart there are some cells which synthesize fat and that their peculiar distribution denoted a relationship to the Purkinje system. More recently Kuwabara and Cogan (1959) have confirmed in the sheep heart that the Purkinje fibres synthesize fat. Chemical analysis of other tissues from which considerable quantities of fat may be obtained, have indicated that this fat consists predominently of triglycerides in which the fatty acids of the medium are incorporated (Kuwabara and Cogan, 1959).

In our present investigation fat is found to occur in considerable amount as droplets in the immediate vicinity of the bundle of His (Figs. 1, 2 and 3) ; and that too invariably near the blood vessels. So the probability is that, that the fat formed in the Purkinje fibers is given out to the heart muscle where it is utilized for energy. In man and dog the AV node and the bundle of His have been shown to be in direct relationship with a profuse capillary network (Lumb *et al,* 1959). Bing (1956) has pointed out that it is likely that the fatty acids are stored by the heart after elevation in blood fatty acid level after a high fat intake. So it is also probable that the bundle of His takes the fatty acids from the blood and converts them into triglycerides. It should be mentioned here that electron microscopic studies of the bundle of His with special reference to microsomes should be of considerable interest since it is now more or less well established that microsomes are sites for lipid synthesis too (Green, 1959).

Summary

1. A histochemical study of the concentration and localization of lipase, succinic dehydrogenase, cytochrome oxidase, fat, glycogen and mitochondria in the bundle of His and the myocardium of the sheep heart, was carried out.

2. The considerably high concentration of lipase in the Purkinje fibres is correlated with their lipogenetic function and the possible role of lipase in the bundle, is discussed.

Acknowledgement

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OBSERVATIONS ON CHLORIDE CONTENT IN GASTRIC SAMPLES AFTER INGESTION OF EGG PREPARATIONS IN NORMAL HUMAN SUBJECTS

M. L. PAI

I.C.M.R. Inquiry, Department of Physiology, Medical College, Baroda, India.

RESULTS of gastric secretory function by fractional gastric analysis method with alcohol meal have been previously reported from this laboratory (Pai, 1956, 1957 & 1958). In a similar study, the chloride content of the gastric samples withdrawn in response to preparations of eggs ingested was determined, the results of which are presented in this communication. It may be stated that so far the determinations regarding the chloride content in gastric samples that have been done by various workers are after the response to standard test meals, like alcohol meal or toast meal and likewise. Here in the present study an attempt has been made to study the response after various preparations of eggs administered in normal subjects.

Materials and Methods

Normal healthy male medical students of age varying between 18 and 24 years were chosen as subjects for the investigations. Meals of alcohol, and various egg preparations were fed to three subjects. The procedure followed for withdrawing the gastric samples after ingestion of food was the same as adopted earlier (Pai, 1956). The total chloride content in gastric samples was estimated by the modified method of Whitehorn-Volhard (Cole, 1949), and it was calculated in terms of mg. of chlorides per 100 cc. of the stomach contents. The results are shown in the Table No. 1.

Results and Discussions

Several factors may operate under normal and abnormal circumstances to modify the state in which hydrochloric acid exists in the stomach. A portion may remain in the free state, a portion may combine with proteins present in the stomach and a variable portion may exist in inorganic combination. In the event of excessive regurgitation of duodenal contents into the stomach or excessive alkaline secretion of the pyloric

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TABLE No. I

Table showing the total chloride content in mg. per 100 cc. of gastric juice after various, articles of food in normal human subjects

	Gastric samples										
Name of the Article	Fasting	Ist	IInd	IIIrd	IVth	Vth	VIth	VIIth	VIIIth	IXth	Xth
Alcohol 7% 50 cc.	380.0	100.0	200.0	180.0	260.0	260.0	—	—	—	—	—
do	390.0	150.0	170.0	170.0	170.0	220.0	—	—	—	—	—
Raw eggs 100 gms.	—	350.0	380.0	430.0	460.0	—	340.0	290.0	—	—	—
do	—	390.0	410.2	430.0	410.0	350.0	360.0	330.0	300.0	310.0	290.0
Omelette (Two eggs)	—	410.0	420.0	—	—	350.0	325.0	240.0	220.0	—	—
do	—	320.0	340.0	—	480.0	370.0	290.0	220.0	175.0	—	—
Raw eggs (five)	—	—	—	400.0	410.0	360.0	440.0	320.0	270.0	240.0	160.0
Eggs (three) shaken in 100 cc Milk	—	240.0	260.0	260.0	260.0	300.0	330.0	285.0	335.0	300.0	—
do	—	—	—	290.0	300.0	310.0	310.0	310.0	—	—	—
Fried eggs (two)	—	—	—	370.0	370.0	380.0	330.0	300.0	330.0	—	—
do	—	—	370.0	380.0	390.0	440.0	380.0	330.0	360.0	300.0	350.0
Boiled eggs (three)	—	230.0	210.0	290.0	340.0	360.0	350.0	320.0	340.0	—	—

glands, a large proportion of the secreted hydrochloric acid may be neutralized, and under such circumstances neither the curve of free hydrochloric acid nor that of total acidity can be regarded as indicating the true state of gastric secretory activity. Bolton and Goodhart (1933) have emphasized the fact that the curve of total chloride is more nearly representative of the true state of gastric secretory activity since it includes, in addition to free hydrochloric acid and hydrochloric acid combined with protein, salts of hydrochloric acid which are neutral in reaction. It has frequently been observed that the total chloride curve may continue to rise for a variable period after the acid curve has begun to fall, indicating that hydrochloric acid is still being secreted but is being neutralized. The determination of total chloride in the gastric content is particularly valuable in the differentiation between true and false achlorhydria; in true achlorhydria no free hydrochloric acid appears in the gastric contents during the digestive cycle and the curve of total chloride is correspondingly low; in false achlorhydria a normal amount of hydrochloric acid may be secreted but is not present in the free state in the gastric contents because of excessive neutralization, and under such circumstances the curve of total chloride may be essentially normal.

As can be seen from the table, the total chlorides were averagely higher with the egg preparations except the egg-in-milk and the boiled eggs, than with the alcohol meal. In case of the alcohol meal, the fasting stomach contents gave averagely highest chloride readings amongst the corresponding readings obtained in other samples of the same fractional examination. The average chloride reading in the fasting stomach contents was observed to be 385.0 mg. per 100 cc. This falls within the normal range as given by Cantarow and Trumper (1955), where they state that the total chloride content of the fasting juice of normal subjects range from 160.0 to 550.0 mg. per 100 cc. or 45.0 to 155.0 milli equivalents per liter. They also state that the total chlorides vary within narrower limits than the free acidity. Omelette (two eggs) gave on the average the highest readings while the egg-in-milk gave the lowest readings for the total chloride content. For raw eggs (100 gms.), the average highest reading for the total chloride content was 435.0 mg. per 100 cc., while for the same article but given in numbers of five, the highest reading was 440 mg. per 100 cc. ; for boiled eggs given in numbers of three, the average highest total chloride content was 360.0 mg. per 100 cc., for eggs (two) fried in ghee the average highest reading was 410.0 mg. per 100 cc., while for omelette (two eggs) the corresponding reading was 480.0 mg. per 100 cc. Lastly for eggs (three) shaken in milk (100 cc.) the average highest total choride content was 320.0 mg per 100 cc.

The highest chloride readings were obtained between 1–00 and 3–00 hours after the intake of the articles in all the cases of the preparations of eggs except in the case of raw eggs where the highest reading was obtained at 3–15 hours after intake. This was not so, however, in case of the alcohol meal where the highest reading was obtained with the fasting stomach contents while the the total chloride content readings were lower in the subsequent samples. It may be pointed out here that the acidity readings in the fasting samples were also higher than those in subsequent samples. In general the chloride graphs in case of the egg preparations resembled the acid curves.

Summary

(1) The total chloride was estimated in the gastric samples withdrawn from normal human stomachs in reponse to foods like eggs and their preparations.

(2) These were compared with the chloride content in gastric samples obtained after alcohol meal.

(3) The peak of the highest chloride content was obtained between 1 and 3 hrs. in case of all preparations of eggs except raw eggs where it was at 3–15 hrs. The chloride content readings after alcohol meal were lower in other samples than those in their fasting one.

(4) The curves for acidity and for chloride content were found to resemble each other well.

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SHORT COMMUNICATIONS

THE ORIGIN AND INSERTION OF THE PECTORALIS MINOR MUSCLE IN MAN

K. N. Bhargava, K. P. Mehra

Department of Anatomy, Gandhi Medical College, Bhopal, India

and

S. N. Bhargava

Department of Anatomy, G. R. Medical College, Gwalior, India

ON a perusal of the successive editions of Text-Books in Anatomy, it is noticed that the description of the origin of the *pectoralis minor* muscle has not remained exactly the same in all of them. Authors *viz.* Brash (1951), Schaeffer (1953), Johnston (1958) and Grant (1958) have recognized the fact that this muscle very often takes origin from the second rib and sometimes from the first intercostal space or the first rib, in addition to the 3rd, 4th and 5th ribs. Variations in the mode of insertion of this muscle have also been brought to light. Its main insertion is on the coracoid process, but frequently also on the conjoint tendon of the *biceps* and *coraco-brachialis*. Occasionally a part or the entire tendon of the *pectoralis minor* passes over the coracoid process (separated from it by a bursa) to pierce the coraco-acromial ligament to be attached to the capsule of the shoulder joint. All these seem to indicate an evolutionary trend. A survey of the literature has shown that so far not much has been said regarding the morphological status and the functional significance of these variations. Records of these variations with regard to race and sex are also few. Seib (1938) compared the origin and insertion of this muscle in American Whites and Negroes. Anson and his associates (1938) noted the variations in the origin of this muscle but not with respect to sex or race. It is often observed in the dissection hall that in asthenic type of persons with a long sternum, the muscle is usually found to be more cephlic in origin.

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With the above points in view it was decided to investigate the origin and insertion of this muscle in the cadavers dissected during the routine course of dissections. A comparative study of the muscle in higher vertebrates was also undertaken.

The findings in man are based on dissections of the *pectoralis minor* performed on 250 cadavers during the years 1956-59. The muscle was seen to arise from the upper ribs near their costochondral junction and also from the fascia covering the inter-costal spaces. The observations are grouped as follows :

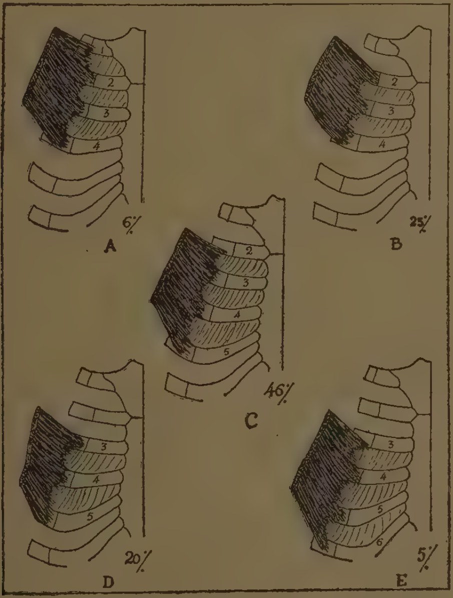

Fig. 1. Showing the variation in the origin of the *pectoralis minor* muscle in man.

A. Origin from 1st, 2nd, 3rd, and 4th ribs and intervening intercostal spaces was recorded in 6% of cases.

B. Origin from 2nd, 3rd, 4th ribs and intercostal spaces was found to be in about 23% of cases.

C. Origin from 2nd, 3rd, 4th, 5th ribs and intervening spaces in 46% of cases.

D. Origin from 3rd, 4th and 5th ribs and intercostal spaces in 20% of cases.

E. Origin from 3rd, 4th, 5th and 6th ribs and intervening spaces was recorded only in 5% cases.

From the above observations it is evident that the classical description of the origin of this muscle *viz.* from 3rd, 4th, 5th, ribs and intervning spaces is true only for a minority (20%).

The cephalic extension of the origin of the muscle in a much larger number of cases in this series in comparison to the findings of Makhani (1957) might be due to the fact that a larger proportion of the cadavers came from scheduled tribes like Gond, Gokhrus etc. inhabiting this part of the country. All except one case in group I and 75% of group II belonged to the female sex.

In all the cases the index denoting the relationship of the length of the manubrium and corpus sterni *viz.*

$$\frac{\text{Length of manubrium} \times 100}{\text{Length of body}} = \text{Sternal Index}$$

was recorded. The average value of this index as given by Glaister (1950) is 54.3 in European females and 46.2 in European males. In groups 1 and 2 the average value was found to be 78 and in groups 3, 4, 5 it was 58 in females and 54 in males.

A comparative study of the pectoral muscles carried out in higher vertebrates by one of us (Bhargava, 1957) indicated certain interesting features. The *pectoralis minor* muscle in many ungulates such as sheep, goat, and pig is represented by two muscles *viz.* (1) an anterior deep pectoral and (2) a post deep pectoral. Both the portions take their origin from the sternum as well as the costal cartilages and are inserted on the humerus. In the carnivora (dog, domestic cat, wild cat and lynx) a part of the muscle takes origin from the sternum and a part from the costal cartilages and like the ungulates is inserted on the head of the humerus. In dog, the deep pectoral muscle corresponding to the *pectoralis minor* is quite extensive.

Amongst the primates as stated by Gregory (1950) even in Gorilla the *pectoralis minor* is represented in two parts, *viz.* 1. *pectoralis minor* (proper) which takes origin from the distal bony part of the 4th and 5th ribs and the intervening fascia and is inserted by a round tendon into the extremity of the coracoid process of the scapula and (2) *pectoralis quartus* muscle which takes its origin from costal cartilages of the 5th, 6th, 7th, 8th ribs and is inserted in the coracoid. In monkey the origin of the muscle has undergone considerable cephalic shift and has a close resemblance to the condition in man. It arises from the outer surfaces of the 3rd, 4th and 5th ribs near their cartilages and is attached to the coracoid process (Bhargava, '57).

Comments

From the above observations it is clear that the degree of development and the extent of the muscle on the ventral aspect of the chest varies much from animal to animal depending on the nature of the movements the limbs are subjected to. Thus one might expect that with the climbing and brachiating mode of progression and the pectorals coming more and more in use they not only shifted their origin from the sternum to the costal cartilages, but their insertion too got secondarily shifted from the humerus to the coracoid process—an acquisition which greatly assisted the arboreal primates in their climbing and brachiating habits. With the broadening of the chest and the erect posture in man, the *pectoralis minor* shifted its origin more laterally and its insertion also got detached from the humerus to be inserted on the coracoid process. Morphologically the coraco-humeral ligament may be regarded as part of the divorced tendon of the *pectoralis minor* going to the humerus (Grant 1958). Considering from a functional view point the cephalic shift of the *pectoralis minor* in man should assist the *serratus anterior muscle* to draw the scapula forwards round the chest wall more powerfully and elegantly—an action of great importance in man and primates when the arm is fixed. Moreover a *pectoralis minor* attached to the 2nd, 3rd, and 4th ribs should also assist the elevation of these ribs and will effect forced respiration more efficiently. At the same time the cephalic shift of the muscle should considerably diminish its action as a depressor of the shoulder. The relative value of such a movement in man is rather insignificant.

The present sample of cadavers is not large enough to subject the observations to statistical analysis but certain interesting points are brought to light. The fact that the authors of text books, presumbaly on the basis of data collected from cadavers belonging to the white races have noted in recent times a cephalic shifting of the origin of *pectoralis minor*, tend to indicate an evolutionary trend. Such a suggestion is most unwarranted in view of the fact that the so called primitive races show a larger percentage of cases in which this muscle has a higher origin. Another important point worthy of mention is that the sternal index cannot be used as a reliable factor in the determination of sex. The average sternal index of 54 for males, which is much higher than the average figure of 46 for males (according to Glaister, 1950) is actually very near the sternal index for females. Our figures for the sternal index namely 54 for males and 58 for females tally with those obtained by Narain and Verma (1958).

Summary

The cephalic shifting in the *pectoralis minor* muscle in man was found to be associated with a higher sternal index. Female cadavers exhibited a higher origin of the muscle in a larger percentage of cases perhaps due to similar reasons.

The functional advantage of the cephalic shifting of the muscle is discussed.

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EFFECT OF INJURY AT SHOULDER JOINT ON THE TWO TYPES OF FIBRES OF THE PIGEON BREAST MUSCLE—A CASE STUDY

R. M. NAIK

Department of Zoology, M. S. University, Baroda, India

THE pigeon breast muscle, *pectoralis major*, is a mixed muscle consisting of two types of fibres, the narrow red and the broad white fibres. In the studies conducted in our laboratories and reviewed recently by George and Naik (1959), these two types of fibres have been shown to be histologically and physiologically distinct types. In such a mixed type of muscle therefore, the changes brought about in its constituent fibres under conditions inducing atrophy of the muscle, should be of considerable interest. George and Naik (1959) have shown that in disuse atrophy of the *pectoralis major* muscle of the pigeon, when induced experimentally by restricting the wing movements for three months by means of a plaster cast at the shoulder joint, the white fibres showed great reduction in size, whereas the red fibres were affected only to a slight extent. A pigeon that was injured at the shoulder joint on one side, happened to come into my hands and on examining its breast muscles the present observations were made.

The pigeon was purchased along with several others from a local dealer with no previous knowledge of its having been injured. On killing it and examining a fresh frozen section of a piece of the left breast muscle, during my studies of the bird flight muscles, I was astonished to find that all the white fibres appeared extremely reduced in size whereas the red fibres were almost normal. An examination of the breast muscle of the other side showed that both the types of fibres were normal or atleast no abnormality was detectable. The remaining portions of the muscle from both sides were then fixed in 10% formalin for further histological observations. On examining the carcass, a healed up wound at left shoulder joint was noticed. The cause of the injury could not be ascertained but however, the nature and the extent of the damage caused, suggested that it might have been inflicted by a bullet that brushed against the shoulder obliquely. On reflecting the skin a deep gutter wound extending from the surface of the body to ~~acetabulum~~ Glenoid cavity could be detected. Some part of the

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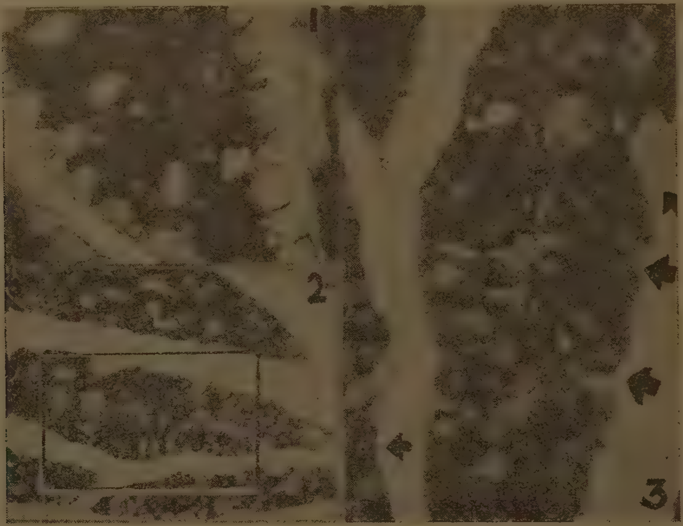
humerus and scapula proximal to the glenoid cavity ~~acetabulum~~ were broken to pieces and nearly all the muscles around the wound showed acute fibrosis. There was no sign of injury on the breast muscle. I have no information on the flying abilities of this pigeon but the nature of the injury suggests that the movements of the left wing at the shoulder joint must have been considerably restricted. On the other hand the right shoulder joint appeared quite normal.



Figs. 1-3. Microphotographs of the transverse sections of the pigeon breast muscles. Sudan Black B.

1. The normal breast muscle of the right side. X100.
2. Left pectoralis showing signs of atrophy. X100.
3. Magnified view of the portion marked by rectangle in fig. 2. White fibres are shown with arrows.

The frozen sections cut out from the muscle pieces fixed in formalin and embedded in gelatin, were stained with Sudan Black B and microphotographed (figures 1-3). In comparison to the white fibres the red fibres in both the pectoralis showed greater affinity for Sudan Black as is the case with normal pigeons. Figure 1 represents the t.s. of the right pectoralis, where the two types of fibres appear normal. On the other hand, in figure 2 and 3, presenting the structure of the left pectoralis, nearly all the white fibres show great reduction in size whereas the red fibres are only slightly reduced.

These observations support the findings of George and Naik (1959) that, compared to the red fibres, the white fibres are more susceptible to disuse atrophy induced by restricting the movements of the wing at the shoulder joint.

Summary

In a pigeon that was injured at the left shoulder joint, probably by a bullet shot, the left breast muscle showed signs of disuse atrophy in that all the white fibres showed great reduction in size, whereas the red ones were only slightly reduced. On the other hand both the types of fibres in the right breast muscle appeared normal.

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I am grateful to Professor J. C. George for taking a keen interest in this work.

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FREE AMINO ACIDS IN THE BLOOD OF SOME REPTILES AND MAMMALS,

S. G. Nair

Veterinary College, Trichur, Kerala, India.

THE free amino acids of the blood, like glucose and fats are nutritive substances on their way to the tissues. In man the amount of amino acids in the blood varies between 4.8–7.8 mg. Amino acids, besides being a source of nutritive material out of which the proteins of the body are built up, also regulate the osmolar concentration of the blood. Liver is directly concerned with the proper maintenance of amino acid level in the blood and the removal of this organ raises the free amino acid level of blood (Flock 1951).

Very little information is available regarding the free amino acid content of the blood of various animals.

Material and Methods.

The free amino acid content of the blood of the following reptiles and mammals was determined.

Reptiles	*Mammals*
1. *Varanus griseus*	1. *Suncus caeruleus*
2. *Ptyas mucosus*	2. *Felis domesticus*
3. *Naja naja*	3. *Pteropus giganteus*
4. *Vipera russelli*	4. *Macaca radiata*
5. *Distira cyanocincya*	
6. *Natrix piscator*	
7. *Lissemys punctata*	
8. *Geomyda trijuga*	

The protein free blood filterate for the analysis was obtained by the method of Folin and Wu as given by Hawk, *et. al.*, (1949).

The free amino acid content of the blood was estimated by the method given by Hawk, *et al.*, (1949).

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Results

Total free amino acids in the blood of the animals examined

	Reptiles	mg. per 100 ml. of blood
1.	Varanus	9.795 ± 0.753
2.	Lissemys	7.224 ± 0.326
3.	Geomyda	12.542 ± 0.246
4.	Ptyas	9.994 ± 0.463
5.	Vipera	8.788 ± 0.042
6.	Naja	8.829 ± 0.086
7.	Distira	12.648 ± 1.756
8.	Natrix	10.333 ± 1.882
	Mammals	
1.	Suncus	14.138 ± 4.556
2.	Pteropus	9.043 ± 1.043
3.	Felis	10.323 ± 0.441
4.	Macaca	7.070 ± 0.237

Discussion

Among the animals worked out no marked differences between the reptiles and mammals in the free amino acid content of the blood could be noticed.

Among reptiles as well as mammals there is considerable variation. Thus among the reptiles the free amino acids in the blood of Lissemys amounts to 7.224 ± 0.326 while that of Geomyda, another chelonian is 12.542 ± 0.246. Among mammals Macaca shows a value of 7.070 ± 0.237, while that of Suncus is 14.138 ± 4.556. The value obtained for Macaca compares favourably with that for man which varies between 4.8 and 7.8 (Meyers, 1924).

The high value obtained for Suncus (14.138 ± 4.556) may be due to its high metabolic rate and the high protein content in its diet, being an insectivore. In this connection it may be mentioned that this animal has a very high N. P. N. content in its blood—111.307 ± 24.542 (Nair, 1955).

Summary

No significant differences between reptiles and mammals could be noticed in the free amino acid content of their blood in both groups. In the both groups there are animals with high and low values.

Acknowledgement

My greatful thanks are due to Dr. C. J. George for the suggestion of the problem and for guidance. I am also indebted to the Director, Haffkine Institute for kindly supplying the shrews, and to the authorities of Taraporevala aquarium, Bombay for supplying the sea snakes.

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REVIEWS

Fundamentals of Ornithology—By JOSSELYN VAN TYNE and ANDREW J. BERGER, (1959). John Wiley and Sons, Inc., New York 16, xii + 624 pp., 252 illus., $ 11.75.

Van Tyne had for many years toiled towards the presentation in a single volume, a comprehensive treatise on the life of birds, to the modern student of ornithology. But his untimely demise early in 1957 would have deprived the world of the thoughts and ideas of a great scholar had not his friend Andrew Berger fulfilled a solemn promise of completing the work in the event of the former's inability to do so.

Modern ornithology is not merely bird watching as is popularly understood but has grown itself into a great subject comprising of several highly specialized areas where modern techniques of scientific methodology are being increasingly employed. So the task of the authors has been a stupendous one in attempting to present in a single volume a gist of all our present knowledge of the life of birds. One important criticism against this book could be that this attempt to cover in a single volume what should really have been done in two volumes, is rather too ambitious. Such a criticism cannot be justified because the authors' purpose in presenting to a graduate student a basic knowledge of the life of birds is clearly indicated in the title of the book itself and to my mind they have admirably fulfilled their task. We have excellent treatises in bird biology which are more useful to the teacher than to the student. So now, we have for the first time a book which is the only one in the English language if not in any language, giving a comprehensive treatment of the subject for a modern student. It must be also mentioned that the authors have not tied themselves down to giving just the bare fundamentals but have intelligently utilized every opportunity to give an insight to the student into the vast areas of knowledge that still lie unexplored.

The book, however, as all books are in their first appearance, is not without defects. The most striking is that it is out of date by a few years during which time some very exciting and important advances in our knowledge of the biology of birds have been made. The recent studies of the stellar orientation, the use of radar and the problem of energetics in sustained flight in understanding migration, have not been included.

Similarly the discovery of the salt glands in birds is also a conspicuous omission. Looking into the circumstances under which the book was written, these shortcomings are pardonable and could be profitably remedied in the next edition of the book.

" Fundamentals of Ornithology " is a great work which certainly has filled a vacuum that has hitherto existed. In the chapters for which Van Tyne was chiefly responsible, the personality of a great ornithologist that he was, stands high as a cliff while in others that of Berger, the gifted anatomist, is clearly discernible even though one would have wished for a greater mingling of the talents. The excellent illustrations by George Sutton have added lustre and elegance to the book thereby rendering it a thing of beauty. Finally I should like to mention that I am personally interested in this book because it has come at an opportune time when I have been looking for a single book which could be recommended as a text-book at the University of Baroda, for our newly introduced graduate course of Avian Biology which is the first of its kind in India.

J. C. George

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which published (only recognised abbreviations of journals, underline) Volume number (double underline) and page number. *e.g.*

Haines, R. W. 1934 The homologies of the flexor and adductor muscle of the thigh.
J. Morph., **56**, 21.

Titles of papers may be omitted altogether. Books should be listed as follows: Author's last name followed by initials, year of publication, title, publishers and place of publication. *e.g.*

Heilbrunn, L. V. 1952 An Outline of General Physiology.
W. B. Saunders Company, Philadelphia.

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